University of Alberta

THE EFFECTS OF ESTROGEN AND AGING ON VASCULAR PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE AND NITRIC OXIDE SYNTHASE.

by

Ken Gerald Stewart

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Physiology

Edmonton, Alberta

Spring 2000

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-60181-1

Canada

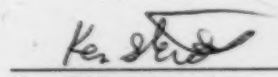
University of Alberta

Library Release Form

Name of Author:	Ken Gerald Stewart
Title of Thesis:	The Effects of Estrogen and Aging on Vascular Prostaglandin-Endoperoxide Synthase and Nitric Oxide Synthase.
Degree:	Master of Science
Year this Degree Granted:	2000

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.



Ken G. Stewart
10824-18 Avenue
Edmonton, Alberta
T6J 6P1

Dated: January 17, 2000

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **The Effects of Estrogen and Aging on Vascular Prostaglandin-Endoperoxide Synthase and Nitric Oxide Synthase**, submitted by **Ken Gerald Stewart** in partial fulfillment of the requirements for the degree of Master of Science.

Dr. S.T. DAVIDGE
Supervisor

Dr. B.F. MITCHELL
Supervisory Committee Member

Dr. D.M. OLSON
Supervisory Committee Member

Dr. D.J. PEHOWICH
Examining Committee Member

Dr. J.J. GREER
Chair, Examining Committee

Dated: January 13, 2000

ABSTRACT

The prostaglandin-endoperoxide synthase (PGHS) and nitric oxide synthase (NOS) pathways play important roles in the regulation of vascular function. Aging is associated with pathological changes in PGHS and NOS-dependent modulation of vessel tone. In particular, aged vessels demonstrate blunted endothelium-dependent relaxation. However, estrogen has been found to enhance NO-dependent relaxation and suppress PGHS-dependent vasoconstriction. Based on these findings, we hypothesized that: estrogen suppresses the production of endothelium-derived vasoconstrictive eicosanoids and enhances NO production; and chronic induction of the PGHS-2 isoform during aging results in blunted endothelium-dependent relaxation. We found that estrogen significantly reduced cultured endothelial cell production of the PGHS-derived eicosanoids, thromboxane A_2 and prostacyclin, in an estrogen receptor-dependent manner. As well, we identified PGHS-2 as the dominant isoform responsible for blunted endothelium-dependent relaxation in mesenteric arteries from aged rats. Thus, a portion of estrogen's vasoprotective effect may be through attenuating PGHS-2-dependent vasoconstriction that becomes more predominant with age.

To my family

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Sandy Davidge, for her support, friendship, and dedication to my education.

Thank you to Dr. Yunlong Zhang for sharing so much of your knowledge and always being there to help.

One of the highlights of graduate school has been collaborating with Dr. Carlos Fernandez-Patron.

I would like to thank my committee members Dr. Peter Mitchell and Dr. David Olson for their advice and contributions.

I am very grateful to Eileen Marco and Sheila McManus for being such a dependable source of help.

To Dr. Jocelynn Cook, thanks for providing technical assistance whenever I asked.

Finally, thank you to my parents for all their support.

TABLE OF CONTENTS

1. Background

1.1. Introduction 1

1.2. Aging and the Vascular System 2

1.3. Oxidative Stress 4

1.4. Antioxidants 5

1.5. Endothelium-Derived PGHS and NOS Pathways 7

1.5.1. Eicosanoids 7

1.5.2. Oxidative Stress and the PGHS Pathway 12

1.5.3. Nitric Oxide 13

1.5.4. Oxidative Stress and the NOS Pathway 16

1.6. Estrogen and the Vascular System 17

1.7. Summary 21

1.8. References 24

2. Estrogen Decreases Prostaglandin-Endoperoxide Synthase (PGHS) Products From Endothelial Cells.

2.1. Introduction 42

2.2. Materials and Methods 43

2.2.1. Reagents 43

2.2.2. Endothelial Cell Culture 44

2.2.3. Nitrite Assay 45

2.2.4. Thromboxane A_2 and Prostacyclin Assays 45

2.2.5. Western Immunoblot 46

2.2.6. Cell Culture Protocol 47

2.2.7. Data Analysis 48

2.3. Results 48

2.3.1 Eicosanoids and Nitric Oxide 48

2.3.2 Tamoxifen 49

2.3.3 Protein Expression 49

2.4. Discussion 50

2.5. References 65

3. Aging Increases PGHS-2 Dependent Vasoconstriction in Rat Mesenteric Arteries.

3.1 Introduction 69

3.2 Methods 71

3.2.1 General Animal Model 71

3.2.2 Experimental Design 72

3.2.3 Data Analysis 74

3.3. Results 74

3.3.1 Vascular Responses 74

3.3.2 Western Immunoblot 76

3.4. Discussion 76

3.5. References 95

4. General Discussion

4.1 Summary 103

4.2 Speculation 107

4.3 Limitations 111

4.3.1. Animal Model 111

4.3.2. Experimental Equipment 112

4.4. Future Directions 112

4.5. Conclusion 116

4.6. References 118

Appendix 128

LIST OF TABLES

Table 2-1. Specificity of thromboxane B_2 enzyme immunoassay kit. 54

Table 2-2. Specificity of 6-keto prostaglandin $F_{1\alpha}$ enzyme immunoassay kit. 54

LIST OF FIGURES

Figure 1-1.	Diagram of the prostaglandin-endoperoxide synthase pathway.	9
Figure 1-2.	Diagram of the nitric oxide and peroxynitrite formation.	14
Figure 1-3.	Summary diagram of experimental objectives.	23
Figure 2-1.	Effect of 17β-estradiol on thromboxane B_2 production in bovine coronary microvascular endothelial cells.	56
Figure 2-2.	Effect of 17β-estradiol on 6-keto prostaglandin $F_{1\alpha}$ production in bovine coronary microvascular endothelial cells.	58
Figure 2-3.	Effect of 17β-estradiol on nitrite production in bovine coronary microvascular endothelial cells.	60
Figure 2-4.	Effect of tamoxifen (10^{-7}M) on thromboxane B_2 production in the absence or presence of log doses of estradiol.	62
Figure 2-5.	Representative Western immunoblots for PGHS-1, PGHS-2, and eNOS in endothelial cells stimulated with 17β-estradiol.	64
Figure 3-1.	EC_{50} values for phenylephrine in young and aged mesenteric arteries.	82
Figure 3-2.	Concentration response curves to methacholine in young and aged rats.	84
Figure 3-3.	Responses of young and aged mesenteric arteries to methacholine in the presence of PGHS pathway inhibitors.	86
Figure 3-4.	Responses of young and aged mesenteric arteries to methacholine in the presence of a NOS inhibitor.	88

Figure 3-5. Concentration-response curves to U-46619 in young and aged mesenteric arteries. 90

Figure 3-6. Concentration-response curves to SNP in young and aged mesenteric arteries 92

Figure 3-7. Western immunoblot bands for PGHS-2 protein expression in young and aged mesenteric arteries. 94

Figure 4-1. Summary of thesis results. 106

LIST OF ABBREVIATIONS

6-keto $PGF_{1\alpha}$	6-keto prostaglandin $F_{1\alpha}$
ACh	acetylcholine
CGRP	calcitonin gene-related peptide
EDRF	endothelium-derived relaxing factor
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
H_2O_2	hydrogen peroxide
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase
L-NMMA	N^G-monomethyl-L-arginine acetate salt
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
OH^-	hydroxyl radical
$ONOO^-$	peroxynitrite
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGHS	prostaglandin-endoperoxide synthase
PGI_2	prostacyclin
PLA_2	phospholipase A_2
PLC	phospholipase C
ROS	reactive oxygen species
SNP	sodium nitroprusside
SOD	superoxide dismutase
TXA_2	thromboxane A_2
TXB_2	thromboxane B_2

1. BACKGROUND

1.1. Introduction

Cardiovascular disease is the number one cause of death in the aging population. However, many of the mechanisms underlying the pathological changes that occur in the vasculature are unknown. Endothelial dysfunction is one of the key factors associated with altered vascular function in aging.[1, 2] Particularly in resistance sized vessels, the endothelium significantly contributes to the regulation of vessel tone by releasing a variety of vasoconstrictors and vasodilators. This balance between vasoconstrictors and vasodilators can be disrupted by factors such as reactive oxidative species (ROS)[3, 4] which become more predominant with age.[5, 6]

Estrogen replacement therapy is one of the effective methods of protecting older women from cardiovascular disease since it reduces the risk by as much as 50%.[7] Although estrogen's beneficial impact on lipid profiles is well established, this mechanism only confers 25-50% of the cardioprotective effect of estrogen.[8] The remaining mechanisms are yet to be determined. Estrogen has been reported to directly affect the endothelium, and specifically influence the nitric oxide synthase (NOS)[9, 10] and prostaglandin-endoperoxide synthase (also known as prostaglandin H synthase, PGHS)[11-13] pathways. Hence, part of estrogen's protective action may be through modulating endothelium-derived NO and PGHS products.

To provide background to the dissertation, I will review how aging and oxidative stress affect the vascular system with emphasis placed on the endothelium-dependent PGHS and NOS pathways. As well, I will summarize some of the effects of estrogen on

the vascular system. This will then lead into the two models we used to further elucidate the effects of aging and estrogen replacement on the vasculature.

1.2. Aging and the Vascular System

Aging is a major independent risk factor for cardiovascular morbidity and mortality.[14] Animal models of aging have demonstrated changes such as increased systolic blood pressure[15] and pulse pressure[16] as well as decreased endothelium-dependent vasorelaxation to muscarinic agonists[1, 16-18] in a variety of arterial vascular beds. Moreover, endothelium-dependent vasodilation decreases steadily with age in healthy humans.[19] However, the mechanisms responsible for the age-related deterioration of vascular function remain poorly understood.

The heterogeneous effect of aging on the vascular system, with respect to both vascular beds and vasoactive pathways, is one of the obstacles in elucidating the processes responsible for an age-dependent decline in vascular function. For example, aging has been found to impair acetylcholine (ACh)-induced relaxation in the aorta but not in the femoral artery.[16] Aging also differentially affects enzyme isoforms. In the aorta, inducible NOS (iNOS) mRNA increases with age, yet endothelial NOS (eNOS) mRNA decreases with age.[20]

Substantial evidence suggests that aging is associated with endothelial dysfunction.[1, 21, 22] In a variety of species and vascular beds, aging is correlated with a blunted relaxation response to the endothelium-dependent agonist ACh.[1, 23-26] Furthermore, aging is associated with enhanced generation and accumulation of superoxide radicals,[5] and increased oxidative damage to protein.[6] The activity[6] and

concentrations[27] of antioxidant enzymes also decrease with age in many tissues. In accord with the above findings, increasing age is associated with decreased metabolic and functional tolerance to oxidative stress in the cardiovascular system.[27, 28] Thus, considering the detrimental effects on the endothelium,[29, 30] it is intuitive that oxidative stress is partly responsible for the age-dependent decline in endothelium-dependent vasorelaxation.

Eicosanoids represent one of the families of vasoactive compounds that contribute to increased vessel tone in aging.[17, 31] In addition to elevated levels of oxidative stress, Davidge *et al.* found aging was associated with blunted endothelium-dependent relaxation and enhanced vasoconstriction that was reversible with PGHS inhibition.[31] Similarly, Koga *et al.* also found that aging resulted in attenuated relaxation to ACh that was a consequence of endothelium-derived eicosanoids.[17]

In physiological conditions, the NOS pathway produces the endothelium-derived vasodilator, nitric oxide (NO), that relaxes the smooth muscle. However, in states of elevated oxidative stress such as aging,[5, 6] ROS can potentially scavenge NO and convert it to the cytotoxic substance peroxynitrite.[4, 32] As well, in some models of aging eNOS activity is suppressed;[33, 34] thus further decreasing the amount of biologically available NO. Indeed, Tschudi *et al.* found that the initial rate of NO release and maximum NO concentration, in the aorta, decrease with age.[35] However, age did not affect these parameters in pulmonary arteries.[35]

1.3. Oxidative Stress

Oxidative stress is a pathological factor associated with aging[5, 6] that can have adverse effects on the vasculature. Described as an imbalance between oxidants and antioxidants,[36] oxidative stress can drastically change the function of the endothelium.[30, 37, 38] It contributes to vascular pathology as a consequence of cytotoxic events such as lipid peroxidation, protein oxidation, and DNA damage.[4] Because some of the species considered part of oxidative stress are not free radicals (ie. hydrogen peroxide (H_2O_2)) and do not oxidize molecules (ie. superoxide is usually a reducing agent[39]), an effective way to describe the substances contributing to oxidative stress is "the total burden of potentially harmful reactive biochemical species that are present in tissues as a consequence of the routine cellular oxidative metabolism of both endogenous and exogenous compounds."[39] The generation of ROS and antioxidant defenses are the two opposing processes that determine the level of oxidative stress in a physiological system.

Endothelial cells, vascular smooth muscle cells, and monocytes are all sources of ROS generation in the vasculature.[39] PGHS, xanthine oxidase, and NADH are examples of enzymes that generate ROS during regular endothelial cell metabolism.[40] Superoxide anion, the product of molecular oxygen receiving an unpaired electron, is a common ROS that is produced under physiological and pathological conditions.[40] During regular metabolism the mitochondrial electron transport chain is a major source of superoxide anions; whereas, pathological levels of NO enhance superoxide anion production by binding cytochrome oxidase and therefore inhibiting mitochondrial respiration.[4]

Although superoxide anions are capable of directly damaging a variety of cellular components, their conversion from a relatively benign state to more reactive species is

most significant. For example, superoxide anions rapidly react with the ubiquitous biological compound NO; thereby consuming a potent vasodilator and forming the highly cytotoxic substance peroxynitrite.[41] They can also be converted to hydrogen peroxide which in turn is responsible for detrimental effects such as inducing upregulation of the apototic signal, Fas, in endothelial cells,[29] inducing aortic contraction,[3] and potentially disrupting cardiovascular homeostasis by increasing endothelial cell permeability.[30] Furthermore, by increasing substrate availability via enhanced phospholipase A_2 (PLA_2) activity in smooth muscle[38] and endothelial cells,[37] hydrogen peroxide can potentially increase production of vasoconstrictive eicosanoids. H_2O_2 generated from superoxide anions and other sources can also be further converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) via the Fenton reaction.[4] Hydroxyl anions are capable of attenuating endothelium-dependent relaxation in arteries.[42]

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \qquad \text{(Fenton reaction)}$$

1.4. Antioxidants

The dramatic impact of oxidative stress on physiological systems is substantiated by the diversity and redundancy of antioxidant systems as well as the high degree of conservation among antioxidant enzymes such as superoxide dismutase (SOD), throughout evolution. Antioxidant defenses can be broken down into three basic categories.[39] One category of antioxidants includes nonenzymatic protein antioxidants such as transferrin, albumin, and ceruloplasmin that primarily function in the plasma.[39] Another category includes nonenzymatic low molecular weight antioxidants that exist in

plasma, intracellular fluid and extracellular fluid, such as lipoproteins, water soluble vitamins, lipid soluble vitamins, and estrogens.[39] Finally, enzymatic antioxidants -the main intracellular defense mechanism- include enzymes such as SOD, catalase, and glutathione peroxidase.

$$2O^{-}_{2} + 2H^{+} \longrightarrow H_2O_2 + O_2 \qquad \text{(superoxide dismutase)}$$

$$2H_2O_2 \longrightarrow O_2 + H_2O \qquad \text{(catalase)}$$

$$ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG \qquad \text{(glutathione peroxidase)}$$

SOD, the enzyme responsible for the conversion of superoxide (O_2^-) to H_2O_2 is present in the cytosol, mitochondria, and extracellular spaces.[43] In most tissues, the intracellular isoforms CuZnSOD[44] and MnSOD,[45] are responsible for the majority of SOD activity.[43] However, in arteries, extracellular SOD (EC-SOD)[46] is conjugated to the vascular wall via heparin sulphate and has an activity level approximately ten-fold greater than in other tissues.[47, 48] Given the enzyme's substantial activity level and localization,[46] extracellular SOD likely exerts a significant antioxidant effect in the vessel wall.[43]

By scavenging superoxide radicals and preventing them from reacting with NO, SOD increases the half life of NO[40] and can therefore enhance vessel relaxation.[49] Because the dismutation of superoxide anions to hydrogen peroxide by SOD is the fastest known enzymatic reaction and SOD exists in high intracellular concentrations of 5-10

μM, it is generally effective at limiting the amount of superoxide anion present.[4] However, when NO concentrations are elevated beyond normal physiological levels, superoxide anions and NO react to form peroxynitrite at a rate three times faster than that of SOD and superoxide.[50]

1.5. Endothelium-Derived PGHS and NOS Pathways

Although a vulnerable target for the damaging effects of oxidative stress and aging, the endothelium plays a fundamental role in regulating vessel function. It is a key barrier between vascular smooth muscle and the contents flowing through the lumen of blood vessels. Stretching over a surface area of approximately $700m^2$,[51] it should be regarded as a distinct endocrine gland.[52] Despite the importance of this tissue, it was only recently recognized as more than the inner lining of a blood vessel. In 1980, Furchgott and Zawadzki demonstrated that the endothelium was essential for ACh induced vasodilation.[53] Since this discovery the endothelium has been shown to release both constricting and relaxing factors.[54] For example, the PGHS pathway mediates both vasoconstriction and vasodilation while the NOS pathway generally relaxes the vasculature.

1.5.1. Eicosanoids

Eicosanoids are a large family of oxygenated C-20 fatty acids that are produced in vascular endothelial[55] and smooth muscle[56] cells. They are synthesized in response to specific stimuli and released immediately.[57] The claim that eicosanoids are local hormones is supported by the facts that plasma concentrations of eicosanoids are usually

too low to be physiologically active[58] and that they are produced in most organs rather than one central location.[57] They mediate a diverse array of physiological events including fever, pain, inflammation, gastric function and vascular function.[59]

Arachidonic acid, the initial substrate for eicosanoid production, is released from glycerophospholipids by enzymes such as PLA_2 and phospholipase C (PLC).[60] One of the potentially rate limiting steps in eicosanoid formation is the conversion of arachidonic acid to the intermediates prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by the enzyme PGHS;[61] which exists in at least two isoforms, PGHS-1 and PGHS-2. PGHS possesses a cyclooxygenase and peroxidase activity that produces PGG_2 and PGH_2 respectively (Figure 1-1).[62] The enzyme is stable for less than 10 minutes and is known for its autoinactivation after approximately 1500 cycles of product formation.[63] It is believed that PGHS inactivation is a consequence of active radical intermediates that are formed during PGHS catalysis.[64] Initially, PGHS-1 was considered to be the constitutive housekeeping isoform, while PGHS-2 was viewed as an inducible inflammatory mediator. However, this classification is not entirely accurate since PGHS-2 is constitutively expressed in some tissues[65, 66] and PGHS-1 expression can be upregulated.[12]

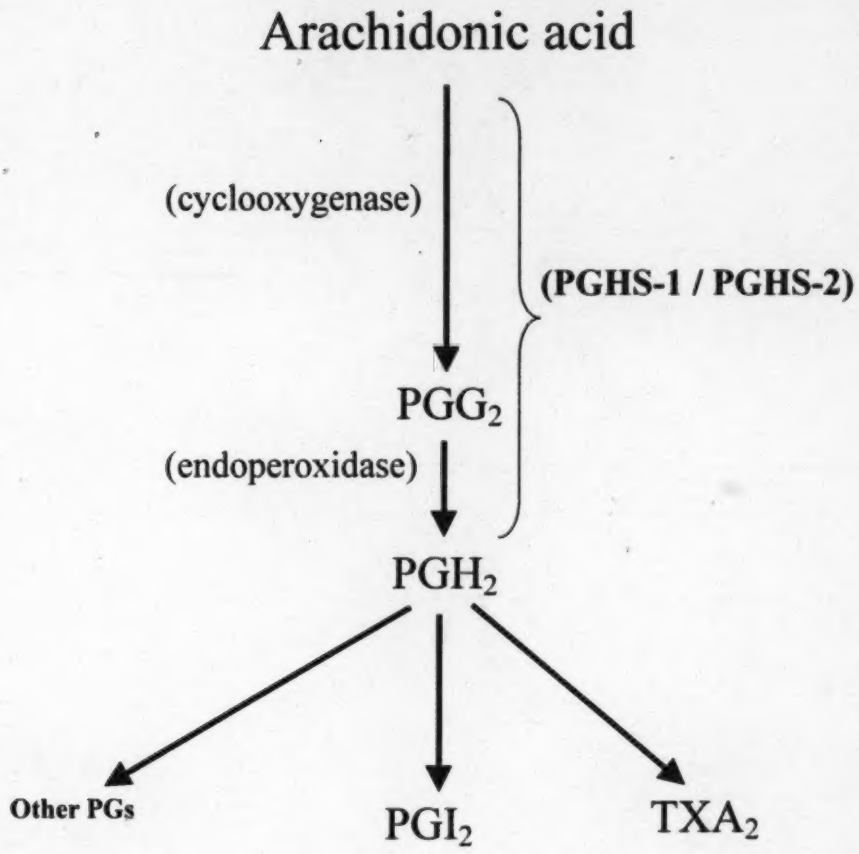


Figure I-1. Summary of the PGHS pathway.

Following the conversion of arachidonic acid to PGH_2, an unstable intermediate with a half-life of about 5 minutes,[67] terminal synthases and isomerases catalyze the synthesis of products including prostacyclin (PGI_2) and thromboxane (TXA_2).[68] Prostacyclin is responsible for vasodilation,[69] decreased platelet aggregation,[70] and reduced smooth muscle cell proliferation.[71] Conversely, TXA_2 induces platelet aggregation, vasoconstriction,[55] and smooth muscle cell proliferation.[72] It is also important to note that PGH_2 is a potent vasoconstrictor that binds to the same receptor as TXA_2.[68, 73]

PGHS-dependent products contribute to pathological vessel function in a number of conditions or models. In a model of hypertension, arteries from spontaneously hypertensive rats (SHR) demonstrate angiotensin II induced vasoconstriction that is PGHS-2 dependent, whereas normotensive vessels from Wistar Kyoto (WKY) rats do not.[74] Similarly, ACh, which facilitates endothelium-dependent relaxation in normotensive rats, stimulates the release of PGHS-1 dependent vasoconstrictors in SHR rats.[75] Alternatively, another study found that an endothelium-derived contracting factor, likely PGH_2, is released in response to ACh in both normotensive and hypertensive rats; however, the effect is much more robust in the hypertensive group.[73] Conditions of oxidative stress, such as the presence of H_2O_2, also cause arterial constriction that is mediated through the PGHS pathway.[3] Preeclampsia, a pathological disease of pregnancy characterized by hypertension, is associated with elevated TXA_2 production as well.[76] Although it has not been extensively studied, aging vessels have been found to display a blunted relaxation response to ACh that is both endothelium and PGHS-dependent.[17, 31] Thus, eicosanoids contribute to increased vessel tone in both

physiological and pathological systems. Finally, in addition to producing vasoconstrictors, the PGHS pathway elevates the level of oxidative stress by producing damaging free radicals as a by-product of catalysis.[77, 78]

In many conditions[3, 73, 79-82] PGH_2 is considered to be the primary eicosanoid responsible for PGHS-dependent constriction since TXA_2/PGH_2 receptor antagonists inhibit contractions, yet TXA_2 synthase inhibitors do not.[73, 80, 82] However, it is still possible that TXA_2 is equally important in causing vessel constriction since blocking the production of TXA_2 could cause an accumulation of PGH_2 and consequently lead to an overestimation of the effect of the pathway intermediate.[82]

Given the pathological effects of PGHS-dependent vasoconstriction it is important to understand the regulation of this pathway. Interestingly, serum from hypertensive patients is capable of increasing production of TXA_2 in human umbilical vein endothelial cells (HUVEC), although the mechanism is not known.[83] Eicosanoid synthesis can be regulated at the levels of substrate availability as well as PGHS expression and/or activity. Factors such as bradykinin,[84] shear stress[85] and endothelin[86] enhance eicosanoid production by increasing substrate availability. In contrast, endothelin-1 induces PGHS-2 protein expression,[86, 87] and IL-1 beta increases both PGHS-2 mRNA and protein expression[72] through the transcription factor NF-KB.[88] Estrogen has been shown to upregulate PGHS-1 expression in vascular endothelial cells.[12] Alternatively, NO is capable of stimulating the activity of PGHS[89-91] through a number of possible mechanisms. By functioning as an oxidizing radical, NO could modify the heme or cyclooxygenase component of PGHS, thereby increasing enzyme

activity.[89, 92] NO may also act indirectly by reacting with superoxide anions to form peroxynitrite, which in turn could ultimately activate PGHS.[93]

1.5.2. Oxidative Stress and the PGHS Pathway

Oxidative stress is another key factor contributing to the regulation of eicosanoid production. For example, exposing aortas to either H_2O_2[94] or ROS generated from xanthine and xanthine oxidase[81] results in increased PGHS-dependent vasoconstriction. The PGHS pathway also contributes to increased vessel tone in ischemic conditions.[95] Such conditions likely enhance production of vasoconstrictive eicosanoids, in part, via the transcription factor NF-KB since it is activated by ROS.[96] NF-KB exists in the cytoplasm as a dimer that is electrostatically complexed with the inhibitory protein IK-B.[88] In the presence of stimuli such as ROS the inhibitory protein is serine-phosphorylated, detached from NF-KB, and degraded; enabling NF-KB to translocate to the nucleus, bind to the promoter region of genes such as PGHS-2, and therefore enhance transcription.[88] As well, H_2O_2 likely enhances eicosanoid production by increasing availability of the substrate, arachidonic acid, through enhanced PLA_2 activity in endothelial[37] and smooth muscle[38] cells.

ROS also interact with free arachidonic acid and nonenzymatically form the constrictive prostaglandin like compounds, isoprostanes.[97] Conditions of oxidative stress such as hypoxia and ischemia reperfusion, facilitate the production of isoprostanes; particularly in the presence of PGHS inhibitors which likely shunt the elevated levels of arachidonic acid[37, 38] away from enzymatic metabolism toward free radical-mediated reactions.[98]

In addition to enhancing the production of vasoconstrictors, ROS further increase vascular tone by decreasing production of the vasodilator, PGI_2. For example, superoxide anions,[99] hydroxyl anions,[100] NO,[100] and peroxynitrite[101] all suppress the activity of prostacyclin synthase. High concentrations of NO can also suppress PGI_2 production by reducing PGHS-2 activity.[102] Hence, oxidative stress contributes to vascular pathology by increasing production of vasoconstrictors while decreasing production of a vasodilator.

1.5.3. Nitric Oxide

The NOS pathway is another one of the fundamental modulators of vascular relaxation (Figure 1-2.). Its importance was not recognized until 1980 when Furchgott and Zawadzki first recognized that endogenous vasodilators such as acetylcholine stimulate the release of an unidentified endothelium-derived relaxing factor (EDRF).[53] The actual identity of EDRF was not determined to be NO until 1987.[103] In addition to regulating vascular tone, the free radical is responsible for inhibiting platelet aggregation and adhesion in the vasculature.[104]

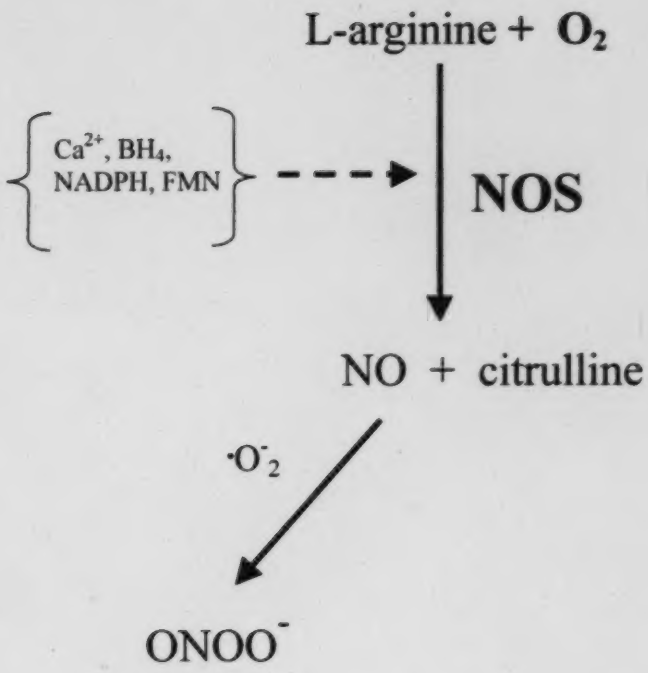


Figure 1-2. Nitric oxide and peroxynitrite formation.

Nitric oxide is formed as a result of the five electron oxidation of a guanidino nitrogen of L-arginine by NOS.[105] The NOS enzyme exists in at least three isoforms which are transcribed from separate genes.[106] With respect to the endothelium, the constitutive and calcium-dependent eNOS (type III) isoform is most predominant.[107] Endothelial NOS maintains basal levels of NO by continuously producing relatively small amounts of product.[107, 108] Neuronal NOS (nNOS; type I) is another example of a calcium-dependent and constitutively expressed isoform.[107] Finally, iNOS (type II) is the calcium-independent and inducible isoform. It is induced by inflammatory mediators and is responsible for producing large amounts of NO that can often be pathological.[41]

Nitric oxide, a ubiquitous intracellular messenger that freely diffuses throughout tissues, is implicated in a diverse array of physiological processes that are constantly being discovered. However, there are three reactions that are fundamental to the effects of NO. First, NO diffusing into the vessel lumen has a limited life span because it is inactivated by red blood cells containing oxyhaemoglobin and converted to nitrate and methemoglobin.[41] Second, superoxide anions produced during various metabolic processes can react with NO to form the highly cytotoxic substance peroxynitrite.[41] Third, many of the functional effects of NO are a consequence of guanylate cyclase activation.[109]

In the vasculature, NO production is modulated in a complex manner by a number of factors. Shear stress, resulting from the movement of fluid through the vessel lumen, is viewed as one of the most important stimuli for NO production.[107] When the flow rate increases or a vessel constricts the level of shear stress increases and consequently enhances NO release.[110] Hence, NO is capable of reversing increases in myogenic tone

by reacting to changes in shear stress. In addition, estrogen,[111] alkaline pH[112] and increased intracellular calcium concentration[113] are capable of increasing NO production. Conversely, by acting through a negative feedback mechanism NO is capable of attenuating NOS activity.[114]

1.5.4. Oxidative Stress and the NOS Pathway

The reciprocal interaction between NO and ROS in the vasculature is complex. In some conditions NO serves a protective role by reducing ROS-induced cell death in mesencephalic dopaminergic cells and lung fibroblasts.[115] It has also been found to improve coronary flow in an ischemic heart model[116] and protect endothelial cells from the damaging effects of H_2O_2 by increasing activity of the antioxidant enzyme, heme oxygenase.[117]

Conversely, when antioxidant reserves such as glutathione are depleted, the potentially cytotoxic effects of NO are revealed.[118] Because superoxide anions react with elevated levels of NO at a rate that greatly exceeds the ability of SOD to scavenge the free radicals, reactive nitrogen species such as peroxynitrite are a significant source of NO-related cytotoxicity.[119] Peroxynitrite exerts its pathological influence by oxidizing targets such as iron/sulfur centers, zinc fingers and protein thiols.[41] The exceptional stability of peroxynitrite further enhances its toxicity by permitting it to diffuse throughout cells.[41] The reaction of NO with oxygen to form other damaging reactive nitrogen species such as nitrite and N_2O_3 is also important to note.[118]

In addition to forming cytotoxic compounds, the interaction between ROS and NO suppresses the biological effect of NO. Inhibiting SOD activity, and therefore

elevating superoxide anions levels, results in attenuated NO-mediated vasorelaxation in bovine coronary arteries.[120] Likewise, administration of exogenous SOD enhances NO-dependent relaxation in rat mesenteric arteries.[49]

1.6. Estrogen and the Vascular System

Given the age-associated increase in oxidative stress and the effect of ROS on the NOS and PGHS pathways, it is not surprising that cardiovascular disease is the leading cause of death in North America; accounting for almost 30% of all deaths.[121] However, among post menopausal women, estrogen replacement has been found to reduce the incidence of cardiovascular disease by as much as 50%.[122] Further substantiating the protective effect of estrogen, compared to men, women under 55 years of age (generally premenopausal) have one third the risk of developing cardiovascular disease.[123] In contrast, by 75 years of age (postmenopausal) women have a virtually identical likelihood of developing cardiovascular disease.[123] Although 25-50% of the beneficial effects of estrogen are attributed to altered lipid profile- increased HDL and decreased LDL[8]- the remaining portion of estrogen-dependent cardiovascular effects are poorly understood.

Estrogen receptors (ER) are one of the key mechanisms through which estrogen exerts its influence on the vascular system. They are ligand activated transcription factors and part of a large family of nuclear hormone receptors.[124] The highly conserved nature of estrogen receptors in endothelial cells, in a variety of sites and species, suggests that they are associated with important functions.[125] Moreover, their utility is supported

by the fact that they have been identified in myocardial tissue, vascular smooth muscle cells, and vascular endothelial cells.[123] According to the classical model of steroid hormone receptors, estrogen enters the cell, binds to an ER, induces dissociation of an inhibitory heat shock protein complex, and consequently facilitates the dimerization of receptors.[123] This ligand-ER dimer complex is then capable of regulating gene transcription by binding to estrogen response elements.[124]

Recent evidence suggests that ER-mediated effects of estrogen are much more complex than the classical steroid hormone receptor theory acknowledges. First, it was recently discovered that there are at least two ER isofroms, ER-α and ER-β.[126, 127] The newly discovered ER-β isoform is 96% identical to the clasical ER in the DNA-binding domain and 60% identical in the hormone-binding domain.[126, 127] Despite the great similarity among the two isoforms, they mediate different or even opposite responses depending on the ligand and DNA response element.[128]

Second, ERs exist in locations that are not encompassed by the classical model of steroid hormone receptors. In addition to intracellular ERs being observed in the cytosol[129] and nucleus,[125] the existence of membrane-ERs has been confirmed.[130-133] Using several antibodies that were specific to epitopes spanning the length of the protein, Pappas *et al.* concluded that membrane ERs are modified versions of their intracellular counterparts.[132] As with peptide hormone and growth factor receptors, membrane bound ERs may be connected to several signaling mechanisms.[130] However, many details regarding the role of membrane bound ERs and the pathways they activate remain to be elucidated.

Third, ERs are now known to exert nongenomic effects in addition to the established genomic actions.[130] For example, they are involved in the regulation of rapid ion fluxes across cell membranes as well as activating enzymes and cellular pumps.[130] Watson *et al.* suggest that these two mechanisms are actually complementary, rather than parallel, because the rapid nongenomic changes help prepare cells for the delayed and long lasting genomic effects.[130]

Estrogen protects the vasculature, in part, by reducing the level of oxidative stress through a variety of mechanisms. With respect to endogenous estrogens, 17β-estradiol is the most abundant effective antioxidant[39] and is capable of scavenging ROS[134] and protecting cells from H_2O_2 induced DNA strand breaks.[135] In addition to functioning directly as an antioxidant, estrogen reduces oxidative stress levels by enhancing antioxidant defense mechanisms. For example, it improves vascular function by increasing SOD and catalase activity.[136] As well, estrogen levels in women are positively correlated with activity of the antioxidant enzyme glutathione peroxidase.[137] Estrogen also protects against myocardial dysfunction and malondialdehyde formation (an indicator of oxidative stress) during ischemia reperfusion through a glutathione peroxidase-dependent mechanism.[138]

Estrogen protects the vascular system by exerting a variety of direct effects on ion channels as well. One of the ways that estrogen reduces vascular tone and therefore the resistance of the vasculature is by lowering the concentration of intracellular calcium via reduced influx through plasma membrane calcium channels.[139] It also increases the efflux of potassium ions through their respective channels; thereby hyperpolarizing cells, closing calcium channels, and consequently relaxing vessels.[10]

The influence of estrogen on several vasoactive pathways is another fundamental mechanism of modulating the cardiovascular system. Although vasoactive PGHS pathway products are, in part, regulated by estrogen, there are great discrepancies in the effects depending on the species, tissue, and conditions used in the study. Some studies suggest estrogen is beneficial to the vasculature by enhancing the production of vasodilatory eicosanoids such as PGI_2. For example, supraphysiological doses of estrogen have been reported to increase PGI_2 production, in rat aortic endothelial cells, independent of PGHS-1 and PGI_2 synthase expression.[11] Alternatively, physiological concentrations of estrogen, in ovine fetal pulmonary endothelial cells, induce an increase in prostacyclin production that is associated with elevated PGHS-1 mRNA levels.[12] Meanwhile, other evidence indicates that estrogen protects the vasculature by reducing the production of PGHS-dependent vasoconstrictors such as TXA_2 and PGH_2. A recent study by Davidge *et al.* observed a significant increase in endothelium-dependent relaxation in arteries from estrogen-replaced rats compared to ovariectomized rats that was due to a suppression of PGHS-dependent vasoconstriction.[13]

Estrogen contributes to the regulation of NO-dependent vasodilation through a variety of processes. It elevates NO levels[10] by increasing eNOS expression[140] and enhancing NOS activity (non-genomically).[9] The antioxidant properties of estrogen may also increase the half-life of NO[32] by suppressing the amount of reactive oxidative species that can inactivate it. Conversely, despite evidence for the stimulatory role of estrogen on the NOS pathway, there are reports that estrogen does not stimulate NO production[141] or expression of NOS.[142, 143]

1.7. Summary/ Rationale

In summary, the vascular endothelium plays a critical role in the regulation of vascular function. The NOS and PGHS pathways significantly contribute to the regulation of vessel tone and are substantially influenced by both estrogen and aging. However, many of the mechanisms underlying the beneficial effects of estrogen still remain to be determined. Although estrogen is known to have a variety of effects on the vasculature, the precise mechanisms are still controversial. It is important to elucidate the precise mechanisms of estrogen so that alternative therapeutic agents can be developed that do not increase the risk of cancer and that are acceptable for men.

In addition, the mechanisms responsible for the vascular changes associated with aging are not well defined. To fully understand the cardioprotective effects of estrogen-replacement thereapy, it is necessary to identify the adaptations that occur in the vasculature during aging.

Thus, the specific hypotheses and aims for the two studies incorporated into this thesis were as follows:

Hypothesis I: Estrogen suppresses the production of PGHS products and increases NO synthesis in endothelial cells.

Aim 1: To measure a time course profile with respect to the effect of estrogen on the production of the PGHS products, TXA_2 and PGI_2.

Aim 2: Using the estrogen receptor antagonist, tamoxifen, determine whether or not estrogen is acting through a receptor dependent mechanism.

Aim 3: To measure the effect of estrogen on NO production.

Aim 4: Examine the effect of estrogen on PGHS-1, PGHS-2 and eNOS protein mass.

Hypothesis II: PGHS-dependent vasoconstriction contributes to an age-associated increase in vessel tone.

Aim 1: To determine the effect of aging on methacholine-induced relaxation in mesenteric arteries.

Aim 2: Using specific inhibitors of the PGHS pathway (PGHS-1, PGHS-2, and the PGH_2/TXA_2 receptor), determine the influence of eicosanoids on vascular function in aging.

Aim 4: Assess the role of NO-mediated relaxation in young and aged vessels.

Aim 5: Measure vascular smooth muscle sensitivity to a TXA_2 mimetic (U-46619) and exogenous NO (sodium nitroprusside, SNP).

Aim 5: Examine the effect of aging on PGHS-1, PGHS-2 and eNOS protein mass in arteries.

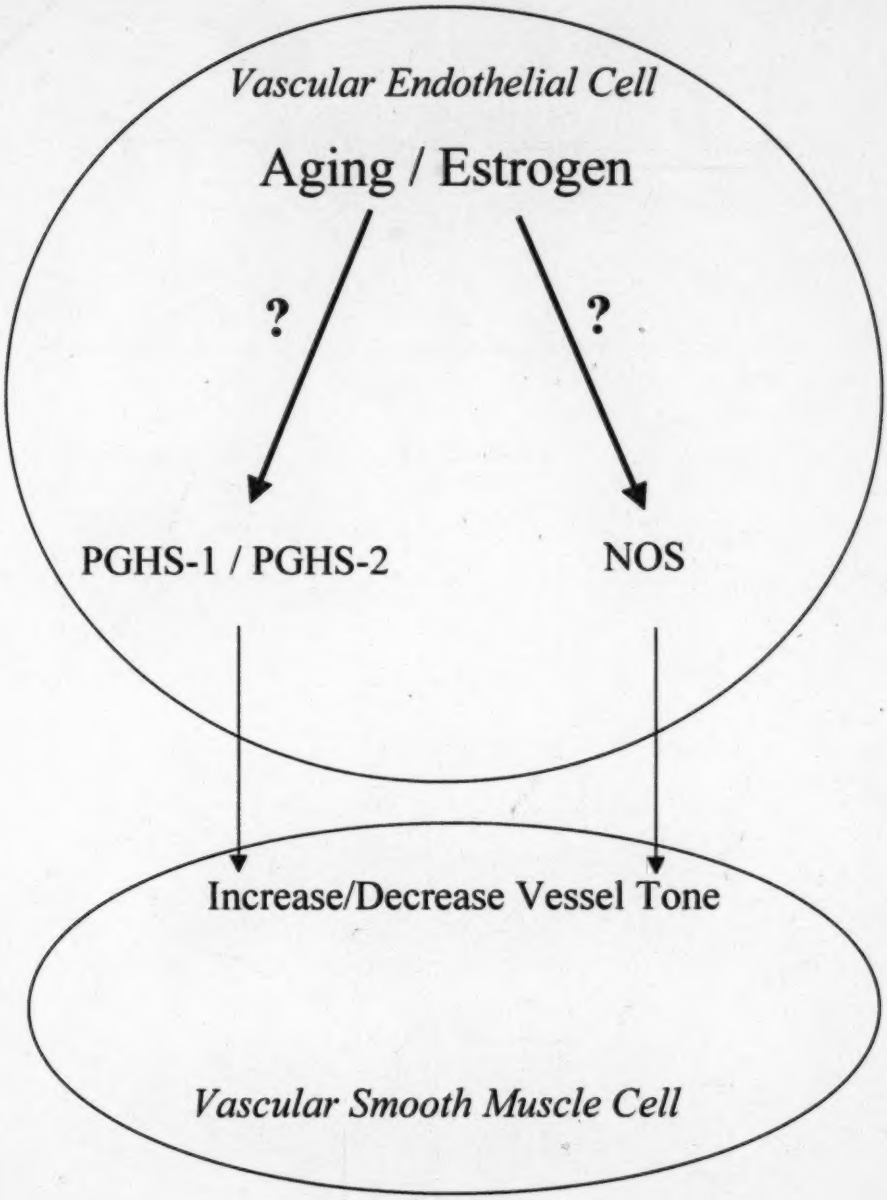


Figure 1-3. Summary of experimental objectives.

1.8. REFERENCES

1. Kung CF, Luscher TF. Different mechanisms of endothelial dysfunction with aging and hypertension in rat aorta. *Hypertension*.1995;25:194-200.
2. Marin J. Age-related changes in vascular responses: a review. *Mech Ageing Dev*.1995;79:71-114.
3. Rodriguez-Martinez MA, Garcia-Cohen EC, Baena AB, Gonzalez R, Salaices M, Marin J. Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanism involved. *Br J Pharmacol*.1998;125:1329-35.
4. Murphy MP, Packer MA, Scarlett JL, Martin SW. Peroxynitrite: a biologically significant oxidant. *Gen Pharmacol*.1998;31:179-86.
5. Susic D. Hypertension, aging, and atherosclerosis. The endothelial interface. *Med Clin North Am*.1997;81:1231-40.
6. Tian L, Cai Q, Wei H. Alterations of antioxidant enzymes and oxidative damage to macromolecules in different organs of rats during aging. *Free Radic Biol Med*.1998;24:1477-84.
7. Fitzpatrick LA. Coronary artery disease in women--an equal opportunity killer. *J Clin Endocrinol Metab*.1998;83:719-20.
8. Barret-Connor E, Bush T. Estrogen and coronary heart disease in women. *J. Am. Med. Assoc*.1991;265:1861-1867.

9. Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, Bender JR. 17-beta estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization. *Circ Res.*1997;81:885-92.

10. Node K, Kitakaze M, Kosaka H, Minamino T, Sato H, Kuzuya T, Hori M. Roles of NO and Ca^{2+}-activated K^{+} channels in coronary vasodilation induced by 17 beta-estradiol in ischemic heart failure. *Faseb J.*1997;11:793-9.

11. Myers SI, Turnage RH, Bartula L, Kalley B, Meng Y. Estrogen increases male rat aortic endothelial cell (RAEC) PGI_2 release. *Prostaglandins Leukot Essent Fatty Acids.*1996;54:403-9.

12. Jun SS, Chen Z, Pace MC, Shaul PW. Estrogen upregulates cyclooxygenase-1 gene expression in ovine fetal pulmonary artery endothelium. *J Clin Invest.*1998;102:176-83.

13. Davidge ST, Zhang Y. Estrogen replacement suppresses a prostaglandin H synthase-dependent vasoconstrictor in rat mesenteric arteries. *Circ. Res.*1998;83:388-395.

14. Frohlich ED, Apstein C, Chobanian AV, Devereux RB, Dustan HP, Dzau V, Fauad-Tarazi F, Horan MJ, Marcus M, Massie B, et al. The heart in hypertension [published erratum appears in N Engl J Med 1992 Dec 10;327(24):1768. *N Engl J Med.*1992;327:998-1008.

15. Soltis EE. Effect of age on blood pressure and membrane-dependent vascular responses in the rat. *Circ Res.*1987;61:889-97.

16. Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S, Luscher TF. Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. *Hypertension*.1997;30:817-24.

17. Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y, Fujishima M. Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat. *Hypertension*.1989;14:542-8.

18. Mantelli L, Amerini S, Ledda F. Roles of nitric oxide and endothelium-derived hyperpolarizing factor in vasorelaxant effect of acetylcholine as influenced by aging and hypertension. *J Cardiovasc Pharmacol*.1995;25:595-602.

19. Gerhard M, Roddy MA, Creager SJ, Creager MA. Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. *Hypertension*.1996;27:849-53.

20. Challah M, Nadaud S, Philippe M, Battle T, Soubrier F, Corman B, Michel JB. Circulating and cellular markers of endothelial dysfunction with aging in rats. *Am J Physiol*.1997;273:H1941-8.

21. Atkinson J. Effect of aging and chronic angiotensin I converting enzyme inhibition on the endothelial function of the mesenteric arterial bed of the rat. *Am J Cardiol*.1995;76:19E-23E.

22. Mayhan WG, Faraci FM, Baumbach GL, Heistad DD. Effects of aging on responses of cerebral arterioles. *Am J Physiol*.1990;258:H1138-43.

23. Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, Sudano I, Salvetti A. Aging and endothelial function in normotensive subjects and patients with essential hypertension. *Circulation*.1995;91:1981-7.

24. Rodriguez-Martinez MA, Alonso MJ, Redondo J, Salaices M, Marin J. Role of lipid peroxidation and the glutathione-dependent antioxidant system in the impairment of endothelium-dependent relaxations with age. *Br J Pharmacol*.1998;123:113-21.

25. Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y, Takeshita A. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation*.1993;88:77-81.

26. Shirasaki Y, Su C, Lee TJ, Kolm P, Cline WH, Jr., Nickols GA. Endothelial modulation of vascular relaxation to nitrovasodilators in aging and hypertension. *J Pharmacol Exp Ther*.1986;239:861-6.

27. Abete P, Napoli C, Santoro G, Ferrara N, Tritto I, Chiariello M, Rengo F, Ambrosio G. Age-related decrease in cardiac tolerance to oxidative stress. *J Mol Cell Cardiol*.1999;31:227-36.

28. Harris NR, Langlois KW. Age-dependent responses of the mesenteric vasculature to ischemia- reperfusion. *Am J Physiol*.1998;274:H1509-15.

29. Suhara T, Fukuo K, Sugimoto T, Morimoto S, Nakahashi T, Hata S, Shimizu M, Ogihara T. Hydrogen peroxide induces up-regulation of Fas in human endothelial cells. *J Immunol*.1998;160:4042-7.

30. McQuaid KE, Smyth EM, Keenan AK. Evidence for modulation of hydrogen peroxide-induced endothelial barrier dysfunction by nitric oxide in vitro. *Eur J Pharmacol*.1996;307:233-41.

31. Davidge ST, Hubel CA, McLaughlin MK. Impairment of vascular function is associated with an age-related increase of lipid peroxidation in rats. *Am J Physiol*.1996;271:R1625-31.

32. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A*.1990;87:1620-4.

33. Cernadas MR, Sanchez de Miguel L, Garcia-Duran M, Gonzalez-Fernandez F, Millas I, Monton M, Rodrigo J, Rico L, Fernandez P, de Frutos T, Rodriguez-Feo JA, Guerra J, Caramelo C, Casado S, Lopez F. Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. *Circ Res*.1998;83:279-86.

34. Chou TC, Yen MH, Li CY, Ding YA. Alterations of nitric oxide synthase expression with aging and hypertension in rats. *Hypertension*.1998;31:643-8.

35. Tschudi MR, Barton M, Bersinger NA, Moreau P, Cosentino F, Noll G, Malinski T, Luscher TF. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. *J Clin Invest*.1996;98:899-905.

36. Shireman PK, Pearce WH. Endothelial cell function: biologic and physiologic functions in health and disease. *Am J Roentgenol*.1996;166:7-13.

37. Boyer CS, Bannenberg GL, Neve EP, Ryrfeldt A, Moldeus P. Evidence for the activation of the signal-responsive phospholipase A_2 by exogenous hydrogen peroxide. *Biochem Pharmacol*.1995;50:753-61.

38. Chakraborti S, Chakraborti T. Down-regulation of protein kinase C attenuates the oxidant hydrogen peroxide-mediated activation of phospholipase A_2 in pulmonary vascular smooth muscle cells. *Cell Signal*.1995;7:75-83.

39. Keaney JF, Jr., Vita JA. Atherosclerosis, oxidative stress, and antioxidant protection in endothelium-derived relaxing factor action. *Prog Cardiovasc Dis*.1995;38:129-54.

40. Katusic ZS. Superoxide anion and endothelial regulation of arterial tone. *Free Radic Biol Med*.1996;20:443-8.

41. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*.1996;271:C1424-37.

42. Todoki K, Okabe E, Kiyose T, Sekishita T, Ito H. Oxygen free radical-mediated selective endothelial dysfunction in isolated coronary artery. *Am J Physiol*.1992;262:H806-12.

43. Luoma JS, Stralin P, Marklund SL, Hiltunen TP, Sarkioja T, Yla-Herttuala S. Expression of extracellular SOD and iNOS in macrophages and smooth muscle cells in human and rabbit atherosclerotic lesions: colocalization with epitopes characteristic of oxidized LDL and peroxynitrite-modified proteins. *Arterioscler Thromb Vasc Biol*.1998;18:157-67.

44. Sherman L, Dafni N, Lieman-Hurwitz J, Groner Y. Nucleotide sequence and expression of human chromosome 21-encoded superoxide dismutase mRNA. *Proc Natl Acad Sci U S A*.1983;80:5465-9.

45. Beck Y, Oren R, Amit B, Levanon A, Gorecki M, Hartman JR. Human Mn superoxide dismutase cDNA sequence. *Nucleic Acids Res*.1987;15:9076.

46. Hjalmarsson K, Marklund SL, Engstrom A, Edlund T. Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. *Proc Natl Acad Sci U S A*.1987;84:6340-4.

47. Stralin P, Karlsson K, Johansson BO, Marklund SL. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. *Arterioscler Thromb Vasc Biol*.1995;15:2032-6.

48. Karlsson K, Marklund SL. Extracellular superoxide dismutase in the vascular system of mammals. *Biochem J*.1988;255:223-8.

49. Davidge ST, Ojimba J, McLaughlin MK. Vascular function in the vitamin E-deprived rat: an interaction between nitric oxide and superoxide anions. *Hypertension*.1998;31:830-5.

50. Huie RE, Padmaja S. The reaction of NO with superoxide. *Free Radic Res Commun*.1993;18:195-9.

51. Luscher TF, Barton M. Biology of the endothelium. *Clin Cardiol*.1997;20:II-3-10.

52. Anggard E. Nitric oxide: mediator, murderer, and medicine. *Lancet*.1994;343:1199-206.

53. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*.1980;288:373-6.

54. Luscher TF. The endothelium. Target and promoter of hypertension? *Hypertension*.1990;15:482-5.

55. Luscher TF, Boulanger CM, Dohi Y, Yang ZH. Endothelium-derived contracting factors. *Hypertension*.1992;19:117-30.

56. Smith WL. The eicosanoids and their biochemical mechanisms of action. *Biochem J.*1989;259:315-24.

57. Smith W. *Annu. Rev. Physl.*1986;48:251-262.

58. Granstrom E, Samuelsson B. Quantitative measurement of prostaglandins and thromboxanes: general considerations. *Adv Prostaglandin Thromboxane Res.*1978;5:1-13.

59. Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, Pick S, Trzaskos JM. Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase. *Proc Natl Acad Sci U S A.*1994;91:11202-6.

60. Gerritsen ME. Physiological and pathophysiological roles of eicosanoids in the microcirculation. *Cardiovasc Res.*1996;32:720-32.

61. DeWitt DL. Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim Biophys Acta.*1991;1083:121-34.

62. Smith WL, Marnett LJ, DeWitt DL. Prostaglandin and thromboxane biosynthesis. *Pharmacol Ther.*1991;49:153-79.

63. Hemler ME, Lands WE. Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J Biol Chem.*1980;255:6253-61.

64. Wu G, Wei C, Kulmacz RJ, Osawa Y, Tsai AL. A mechanistic study of self-inactivation of the peroxidase activity in prostaglandin H synthase-1. *J Biol Chem.*1999;274:9231-7.

65. Ermert L, Ermert M, Althoff A, Merkle M, Grimminger F, Seeger W. Vasoregulatory prostanoid generation proceeds via cyclooxygenase-2 in noninflamed rat lungs. *J Pharmacol Exp Ther.*1998;286:1309-14.

66. Asano K, Lilly CM, Drazen JM. Prostaglandin G/H synthase-2 is the constitutive and dominant isoform in cultured human lung epithelial cells. *Am J Physiol*.1996;271:L126-31.

67. Halushka PV, Mais DE, Mayeux PR, Morinelli TA. Thromboxane, prostaglandin and leukotriene receptors. *Annu Rev Pharmacol Toxicol*.1989;29:213-39.

68. Smith L. *Biochem. J*.1989;259:315-324.

69. Williams TJ. Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *Br J Pharmacol*.1979;65:517-24.

70. Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A_2, and prostacyclin. *Pharmacol Rev*.1978;30:293-331.

71. Hara S, Morishita R, Tone Y, Yokoyama C, Inoue H, Kaneda Y, Ogihara T, Tanabe T. Overexpression of prostacyclin synthase inhibits growth of vascular smooth muscle cells. *Biochem Biophys Res Commun*.1995;216:862-7.

72. Chen G, Kamal M, Hannon R, Warner TD. Regulation of cyclo-oxygenase gene expression in rat smooth muscle cells by catalase. *Biochem Pharmacol*.1998;55:1621-31.

73. Kato T, Iwama Y, Okumura K, Hashimoto H, Ito T, Satake T. Prostaglandin H_2 may be the endothelium-derived contracting factor released by acetylcholine in the aorta of the rat. *Hypertension*.1990;15:475-81.

74. Zerrouk A, Auguet M, Chabrier PE. Augmented endothelium-dependent contraction to angiotensin II in the SHR aorta: role of an inducible cyclooxygenase metabolite. *J Cardiovasc Pharmacol*.1998;31:525-33.

75. Ge T, Hughes H, Junquero DC, Wu KK, Vanhoutte PM, Boulanger CM. Endothelium-dependent contractions are associated with both augmented expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H_2 in the SHR aorta. *Circ Res*.1995;76:1003-10.

76. Johnson RD, Sadovsky Y, Graham C, Anteby EY, Polakoski KL, Huang X, Nelson DM. The expression and activity of prostaglandin H synthase-2 is enhanced in trophoblast from women with preeclampsia. *J Clin Endocrinol Metab*.1997;82:3059-62.

77. Tsai AL, Palmer G, Kulmacz RJ. Prostaglandin H synthase. Kinetics of tyrosyl radical formation and of cyclooxygenase catalysis. *J Biol Chem*.1992;267:17753-9.

78. Lassmann G, Odenwaller R, Curtis JF, DeGray JA, Mason RP, Marnett LJ, Eling TE. Electron spin resonance investigation of tyrosyl radicals of prostaglandin H synthase. Relation to enzyme catalysis [published erratum appears in J Biol Chem 1992 Mar 25;267(9):6449]. *J Biol Chem*.1991;266:20045-55.

79. Ito T, Kato T, Iwama Y, Muramatsu M, Shimizu K, Asano H, Okumura K, Hashimoto H, Satake T. Prostaglandin H_2 as an endothelium-derived contracting factor and its interaction with endothelium-derived nitric oxide. *J Hypertens*.1991;9:729-36.

80. Luscher TF, Vanhoutte PM. Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension*.1986;8:344-8.

81. Auch-Schwelk W, Katusic ZS, Vanhoutte PM. Thromboxane A2 receptor antagonists inhibit endothelium-dependent contractions.

Hypertension.1990;15:699-703.

82. Aksoy MO, Harakal C, Smith JB, Stewart GJ, Zerweck CR. Mediation of bradykinin-induced contraction in canine veins via thromboxane/prostaglandin endoperoxide receptor activation. *Br J Pharmacol*.1990;99:461-6.

83. Ooi BS, Cohen DJ, Chang TH, Tian Y, Papademetrious V. Stimulation of endothelial cell production of vasoconstrictive substances by hypertensive sera. *Am J Hypertens*.1998;11:240-4.

84. Saunders MA, Belvisi MG, Cirino G, Barnes PJ, Warner TD, Mitchell JA. Mechanisms of prostaglandin E_2 release by intact cells expressing cyclooxygenase-2: evidence for a 'two-component' model. *J Pharmacol Exp Ther*.1999;288:1101-6.

85. Okahara K, Sun B, Kambayashi J. Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells. *Arterioscler Thromb Vasc Biol*.1998;18:1922-6.

86. Schramek H, Coroneos E, Dunn MJ. Interactions of the vasoconstrictor peptides, angiotensin II and endothelin-1, with vasodilatory prostaglandins. *Semin Nephrol*.1995;15:195-204.

87. Coroneos E, Kester M, Thomas P, Dunn MJ. Endothelin regulates PGE_2 formation in rat mesangial cells through induction of prostaglandin endoperoxide synthase-2. *Adv Prostaglandin Thromboxane Leukot Res*.1995;23:117-9.

88. Lukiw WJ, Bazan NG. Strong nuclear factor-kappa B-DNA binding parallels cyclooxygenase-2 gene transcription in aging and in sporadic Alzheimer's disease superior temporal lobe neocortex. *J Neurosci Res*.1998;53:583-92.

89. Davidge ST, Baker PN, Laughlin MK, Roberts JM. Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. *Circ Res.*1995;77:274-83.

90. Tetsuka T, Daphna-Iken D, Miller BW, Guan Z, Baier LD, Morrison AR. Nitric oxide amplifies interleukin 1-induced cyclooxygenase-2 expression in rat mesangial cells. *J Clin Invest.*1996;97:2051-6.

91. Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A.*1993;90:7240-4.

92. Smith WL, Marnett LJ. Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim Biophys Acta.*1991;1083:1-17.

93. Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci U S A.*1996;93:15069-74.

94. Rodriguez-Martinez MA, Garcia-Cohen EC, Baena AB, Gonzalez R, Salaices M, Marin J. Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanism involved. *Br J Pharmacol.*1998;125:1329-35.

95. Naylor HL, Shoemaker JK, Brock RW, Hughson RL. Prostaglandin inhibition causes an increase in reactive hyperaemia after ischaemic exercise in human forearm. *Clin Physiol.*1999;19:211-20.

96. Speir E, Shibutani T, Yu ZX, Ferrans V, Epstein SE. Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection. *Circ Res*.1996;79:1143-52.

97. Porter NA, Caldwell SE, Mills KA. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids*.1995;30:277-90.

98. Mobert J, Becker BF. Cyclooxygenase inhibition aggravates ischemia-reperfusion injury in the perfused guinea pig heart: involvement of isoprostanes. *J Am Coll Cardiol*.1998;31:1687-94.

99. Katusic ZS, Vanhoutte PM. Superoxide anion is an endothelium-derived contracting factor. *Am J Physiol*.1989;257:H33-7.

100. Camacho M, Lopez-Belmonte J, Vila L. Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity. *Circ Res*.1998;83:353-65.

101. Zou M, Martin C, Ullrich V. Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem*.1997;378:707-13.

102. Kosonen O, Kankaanranta H, Malo-Ranta U, Ristimaki A, Moilanen E. Inhibition by nitric oxide-releasing compounds of prostacyclin production in human endothelial cells. *Br J Pharmacol*.1998;125:247-54.

103. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*.1987;327:524-6.

104. Butler AR, Flitney FW, Williams DL. NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. *Trends Pharmacol Sci*.1995;16:18-22.

105. Stuehr DJ, Griffith OW. Mammalian nitric oxide synthases. *Adv Enzymol Relat Areas Mol Biol*.1992;65:287-346.

106. Marsden PA, Heng HH, Scherer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC, Schappert KT. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem*.1993;268:17478-88.

107. Busse R, Fleming I. Regulation and functional consequences of endothelial nitric oxide formation. *Ann Med*.1995;27:331-40.

108. Sessa WC. The nitric oxide synthase family of proteins. *J Vasc Res*.1994;31:131-43.

109. Nussler AK, Billiar TR. Inflammation, immunoregulation, and inducible nitric oxide synthase. *J Leukoc Biol*.1993;54:171-8.

110. Griffith TM, Edwards DH. Myogenic autoregulation of flow may be inversely related to endothelium- derived relaxing factor activity [published erratum appears in Am J Physiol 1990 Dec;259(6 Pt 3):following table of contents]. *Am J Physiol*.1990;258:H1171-80.

111. Cicinelli E, Ignarro LJ, Lograno M, Galantino P, Balzano G, Schonauer LM. Circulating levels of nitric oxide in fertile women in relation to the menstrual cycle. *Fertil Steril*.1996;66:1036-8.

112. Fleming I, Hecker M, Busse R. Intracellular alkalinization induced by bradykinin sustains activation of the constitutive nitric oxide synthase in endothelial cells. *Circ Res*.1994;74:1220-6.

113. Newby AC, Henderson AH. Stimulus-secretion coupling in vascular endothelial cells. *Annu Rev Physiol*.1990;52:661-74.

114. Assreuy J, Cunha FQ, Liew FY, Moncada S. Feedback inhibition of nitric oxide synthase activity by nitric oxide. *Br J Pharmacol*.1993;108:833-7.

115. Wink DA, Hanbauer I, Krishna MC, DeGraff W, Gamson J, Mitchell JB. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc Natl Acad Sci U S A*.1993;90:9813-7.

116. Linz W, Wiemer G, Scholkens BA. ACE-inhibition induces NO-formation in cultured bovine endothelial cells and protects isolated ischemic rat hearts. *J Mol Cell Cardiol*.1992;24:909-19.

117. Motterlini R, Foresti R, Intaglietta M, Winslow RM. NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am J Physiol*.1996;270:H107-14.

118. Walker MW, Kinter MT, Roberts RJ, Spitz DR. Nitric oxide-induced cytotoxicity: involvement of cellular resistance to oxidative stress and the role of glutathione in protection. *Pediatr Res*.1995;37:41-9.

119. Tolias CM, McNeil CJ, Kazlauskaite J, Hillhouse EW. Superoxide generation from constitutive nitric oxide synthase in astrocytes in vitro regulates extracellular nitric oxide availability. *Free Radic Biol Med*.1999;26:99-106.

120. Omar HA, Cherry PD, Mortelliti MP, Burke-Wolin T, Wolin MS. Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent and -independent nitrovasodilator relaxation. *Circ Res*.1991;69:601-8.

121. Lerner DJ, Kannel WB. Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. *Am Heart J*.1986;111:383-90.

122. Barrett-Connor E, Bush TL. Estrogen and coronary heart disease in women. *Jama*.1991;265:1861-7.

123. Skafar DF, Xu R, Morales J, Ram J, Sowers JR. Clinical review 91: Female sex hormones and cardiovascular disease in women. *J Clin Endocrinol Metab*.1997;82:3913-8.

124. Grandien K, Berkenstam A, Gustafsson JA. The estrogen receptor gene: promoter organization and expression. *Int J Biochem Cell Biol*.1997;29:1343-69.

125. Venkov CD, Rankin AB, Vaughan DE. Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormone regulation of endothelial function. *Circulation*.1996;94:727-33.

126. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA*.1996;93:5925-30.

127. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett*.1996;392:49-53.

128. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ER alpha and ER beta at AP1 sites. *Science*.1997;277:1508-10.

129. Colburn P, Buonassisi V. Estrogen-binding sites in endothelial cell cultures. *Science*.1978;201:817-9.

130. Watson CS, Gametchu B. Membrane-initiated steroid actions and the proteins that mediate them. *Proc Soc Exp Biol Med*.1999;220:9-19.

131. Brubaker KD, Gay CV. Specific binding of estrogen to osteoclast surfaces [published erratum appears in Biochem Biophys Res Commun 1994 Jul 15;202(1):643]. *Biochem Biophys Res Commun*.1994;200:899-907.

132. Pappas TC, Gametchu B, Watson CS. Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. *FASEB J*.1995;9:404-10.

133. Watson CS, Norfleet AM, Pappas TC, Gametchu B. Rapid actions of estrogens in GH3/B6 pituitary tumor cells via a plasma membrane version of estrogen receptor-alpha. *Steroids*.1999;64:5-13.

134. Subbiah MT. Mechanisms of cardioprotection by estrogens. *Proc Soc Exp Biol Med*.1998;217:23-9.

135. Tang M, Subbiah MT. Estrogens protect against hydrogen peroxide and arachidonic acid induced DNA damage. *Biochim Biophys Acta*.1996;1299:155-9.

136. Ghanam K, Javellaud J, Ea-Kim L, Oudart N. The protective effect of 17-beta estradiol on vasomotor responses of aorta from cholesterol-fed rabbit is reduced by inhibitors of superoxide dismutase and catalase. *Biochem Biophys Res Commun*.1998;249:858-64.

137. Massafra C, De Felice C, Gioia D, Buonocore G. Variations in erythrocyte antioxidant glutathione peroxidase activity during the menstrual cycle. *Clin Endocrinol (Oxf)*.1998;49:63-7.

138. Kim YD, Farhat MY, Myers AK, Kouretas P, DeGroot KW, Pacquing A, Ramwell PW, Suyderhoud JP, Lees DE. 17-beta estradiol regulation of myocardial glutathione and its role in protection against myocardial stunning in dogs. *J Cardiovasc Pharmacol*.1998;32:457-65.

139. Zhang F, Ram JL, Standley PR, Sowers JR. 17 beta-estradiol attenuates voltage-dependent Ca^{2+} currents in A7r5 vascular smooth muscle cell line. *Am J Physiol*.1994;266:C975-80.

140. Hishikawa K, Nakaki T, Marumo T, Suzuki H, Kato R, Saruta T. Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells. *FEBS Lett*.1995;360:291-3.

141. Mikkola T, Ranta V, Orpana A, Ylikorkala O, Viinikka L. Effect of physiological concentrations of estradiol on PGI_2 and NO in endothelial cells. *Maturitas*.1996;25:141-7.

142. Al-Hijji J, Batra S. Downregulation by estrogen of nitric oxide synthase activity in the female rabbit lower urinary tract. *Urology*.1999;53:637-41.

143. Tschugguel W, Zhegu Z, Schneeberger C, Tantscher E, Czerwenka K, Fabry A, Wojta J, Zeillinger R, Huber JC. Estrogen does not induce the calcium-dependent nitric oxide synthase in cultured human uterine endothelial and myometrial smooth muscle cells. *J Vasc Res*.1997;34:281-8.

2. ESTROGEN DECREASES PROSTAGLANDIN H SYNTHASE (PGHS) PRODUCTS FROM ENDOTHELIAL CELLS.

2.1. Introduction

Cardiovascular disease is the number one cause of death in the aging population of North America.[1] Estrogen has been found to reduce the incidence of cardiovascular disease by as much as 50%. Although 25% to 50% of the beneficial effect of estrogen is attributed to altered lipid profile, the remaining portion of estrogen's effect on cardiovascular protection is not well understood.

Estrogen has been found to increase production of the endothelium-derived vasodilator, nitric oxide (NO),[2,3] however its effect on other vasoactive pathways remains to be elucidated. We recently demonstrated that estradiol replacement in ovariectomized rats suppressed endothelium-derived prostaglandin H synthase (PGHS)-dependent vasoconstriction. The enzyme PGHS converts arachidonic acid into the intermediate prostaglandin H_2 (PGH_2), via cyclooxygenase and endoperoxidase activity. Specific synthases act on PGH_2 to produce vasoactive eicosanoids. In the vasculature, prostacyclin (PGI_2) is a vasodilator, whereas thromboxane A_2 (TXA_2) and PGH_2 are potent vasoconstrictors that bind to the same receptor.[4, 5] Indeed, studies of aging[6] and ovariectomy[7] (both conditions relevant to menopausal women) have shown increased PGHS-dependent vasoconstriction acting through the TXA_2/PGH_2 receptor.

Our previous functional study showed that estrogen replacement significantly reduced PGHS-dependent vasoconstriction but did not permit the measurement of

product formation or the determination of many other cellular processes in response to estrogen. We hypothesized that 17β-estradiol inhibits the production of the vasoconstrictive eicosanoid, TXA_2, by acting at the level of PGHS. Hence, the objective of this study was to determine: 1) the time course of TXA_2 production in response to log doses of estradiol; 2) PGHS expression in response to estradiol; 3) the effect of estrogen receptor inhibition on TXA_2 formation; and 4) the effect of estradiol on the NO pathway.

2.2. Materials and Methods

2.2.1. Reagents

L-glutamine, trypsin, α-minimum essential media (α-MEM) with and without phenol red, and horse serum were obtained from GIBCO (Gaithersburg, MD, U.S.A.). Enzyme immunoassay kits for the stable metabolites of TXA_2 and PGI_2 were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Charcoal stripped fetal bovine serum was obtained from Hyclone (Logan, Utah, U.S.A.). Gentamycin, kanamycin, lactate dehydrogenase kits and 17 β-estradiol (water soluble) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2.2.2. Endothelial Cell Culture

A bovine coronary microvascular endothelial cell line, obtained from Gensia Inc, was selected based on the significance of the coronary microvasculature in cardiovascular disease. Moreover, these cells express estrogen receptor protein, endothelial nitric oxide synthase (eNOS) and PGHS as well as produce NO and eicosanoids. Other cellular characteristics include growth as a monolayer, a cobblestone morphology at confluence, positive immunostaining for von Willebrand factor-related antigen, the presence of receptors for acetylated low-density lipoproteins, and secretion of tissue-type plasminogen activator.

Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air with α MEM containing 0.6 mmol/L L-arginine, 10% horse serum, 2 mmol/L L-glutamine, gentamicin (5 μg/ml), and kanamycin (20 μg/ml).

Cells were plated at confluence in six-well tissue culture plates at 5×10^5 cells per mL (1mL per well). After attaching to plates, cells were quiesced in phenol-red free media with charcoal stripped fetal bovine serum for 24 hours before experimental stimulation. Immediately prior to stimulation, this phenol-red free media was replaced with fresh media. Total protein for cells was measured by the Bradford method,[8] with bovine serum albumin used as a standard.

2.2.3. Nitrite Assay

Nitrite, a stable end product of NO, was measured in the culture media by using the spectrophotometric Greiss reaction.[9] An aliquot of medium (180 μL) from each culture well was mixed with 40 μL of Greiss reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid). The mixture was incubated for 10 minutes at room temperature, and the absorbance (optical density, 550nm) was measured in a UVmax kinetic microplate reader (Molecular Devices). Concentrations were determined by comparison with a sodium nitrite standard. The lower limit of detection was 0.2μmol/L.

2.2.4. Thromboxane A_2 and Prostacyclin Assays

TXA_2 and PGI_2 were measured in the cell culture media as their stable metabolites, thromboxane B_2 (TXB_2) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), respectively, by using enzyme immunoassay kits. The lower limit of detection for TXB_2 was 7.8 pg/mL; the lower limit of detection for 6-keto $PGF_{1\alpha}$ was 3.9 pg/mL. All assays contained media controls for the various agents used in the present study to determine that there was no effect of the agents on the assays. Tables 2-1 and 2-2 represent the cross-reactivity of the thromboxane B_2 (TXB_2) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) enzyme immunoassay kits, respectively.

2.2.5. Western Immunoblot

Western immunoblotting was performed for PGHS-1, PGHS-2 and eNOS. For gel electrophoresis, samples were diluted by addition of an equal volume of 2X gel sample buffer (40 mmol/L Tris/Cl pH 6.8, 2% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.02 % bromphenol blue). Samples were boiled for 3 minutes. Equal protein (5 μg) was loaded into individual wells formed within the stacking gel (5% acrylamide in stacking gel buffer, 25 mmol/L Tris-HCl, pH 6.8) overlayed on 10% (PGHS) and 8% (eNOS) acrylamide gels in Tris-HCl, pH 8.8, and separated by electrophoresis at 120 volts for 1.25 hours in a mini-gel apparatus according to the method of Laemmli.[10]

Following separation, samples were transferred to a nylon membrane (Nylon NT, Micron Separations Inc.). Prestained standards (kaleidoscope prestained standards, Bio-Rad) were included in separate lanes in each gel for identification of the approximate molecular weight of unknowns. Ovine PGHS-1 and PGHS-2 standards (Cayman Chemical Co.) and human endothelial cell standard (Transduction Laboratories, Lexington KY, U.S.A.) were added to lanes in the respective gels. Primary monoclonal antibodies (mouse anti-PGHS-1 and anti-PGHS-2, Cayman Chemical Co.; mouse anti-eNOS, Transduction Laboratories) were incubated for 2 h; secondary antibody (polyclonal anti-mouse horseradish peroxidase conjugated, Jackson Immunoresearch, Westgrove PA, U.S.A.) was incubated for 1 hour and ECL detection (Amersham LIFE Science, Oakville ON, Canada) was used. Autoradiography was done using Fuji medical x-ray medical film (Fuji Photo film Co. LTD., Tokyo Japan).

2.2.6. Cell Culture Protocol

After 24 hours of quiescence, the cells were stimulated with log doses of 17 β-estradiol ranging from 0.01 nM to 1.0 nM for 4, 8, or 24 hours. The 17β-estradiol used in this experiment is water soluble as a result of its conjugation with β-cyclodextrin molecules. This method is commonly used to convey aqueous properties to molecules that would otherwise not be soluble. The 17β-estradiol was diluted to the desired concentrations in PBS buffer. Each condition was measured in triplicate wells and in three or four separate experiments. At the end of a stimulation period, the supernatant was collected for measurement of TXB_2, 6-keto-$PGF_{1\alpha}$, and NO_2 formation. The cells were subsequently rinsed with PBS, scraped from the six well plates, and collected in a total volume of 200 μl of a homogenizing buffer. Immediately after collection, the cells were sonicated for approximately 5 seconds.

To determine whether or not estrogen was acting through an estrogen receptor (ER) dependent pathway the ER antagonist, tamoxifen (10^{-7}M), was applied to cells 30 minutes prior to stimulation with 17β-estradiol.

To ensure that cell viability was not affected by treatment conditions, a lactate dehydrogenase assay kit (Sigma) was used.

2.2.7. Data Analysis

Data are summarized as mean ± SEM of at least three individual experiments containing three replicates per condition. A Kruskal-Wallis ANOVA on ranks was used to determine statistical differences among three or more groups. Differences of ranks were considered significant at $P<0.05$. Multiple comparison procedures were performed using Dunnett's test. Differences were considered significant at $P<0.05$.

2.3. Results

All data are expressed as a percentage of the control condition with all products measured first being normalized to protein. Each well contained 5×10^5 cells, as measured by a hemacytometer, when a confluent monolayer had been established. Total protein in each well (≈ 6 μg of protein per well) did not change significantly with stimulation by different agents. Lactate dehydrogenase levels, used as an indicator of cell viability, were consistent among treatment conditions.

2.3.1. Eicosanoids and Nitric Oxide

When the cells were exposed to estrogen for 4 or 8 hours, there was no significant effect on TXB_2 production (Fig. 2-1). However, 24 hour exposure to 0.1nM and 1.0nM estradiol significantly reduced production of TXB_2 ($P< 0.05$) to 67 ± 16% and 69 ± 12% respectively, relative to control (Fig. 2-1). To determine if this inhibition was specific for the vasoconstrictor TXA_2, another PGHS product, 6-keto $PGF_{1\alpha}$, was also measured. As with TXB_2 production, the suppression in 6-keto $PGF_{1\alpha}$ production was not observed until cells were exposed to estradiol for a 24-hour period. 6-keto $PGF_{1\alpha}$ concentrations

were markedly reduced ($p<0.05$) to 35 ± 19% and 17 ± 11% of the control after 24 hours of exposure to 0.1nM and 1.0nM estradiol respectively (Fig. 2-2). With respect to absolute production, the median value for 6-keto $PGF_{1\alpha}$ was 19.7 times greater than TXB_2. The concentration of the NO metabolite, nitrite, was not significantly affected by estradiol (Fig. 2-3).

2.3.2. Tamoxifen

Tamoxifen alone did not alter TXA_2 production relative to control. However, tamoxifen did prevent the inhibitory effect of estradiol on TXA_2 production. When the cells were preincubated with tamoxifen, 24 hour exposure to 0.01nM, 0.1nM and 1.0nM doses of estradiol caused a significant ($P<0.05$) increase of 17.4 ± 3.1%, 26.5 ± 5.7% and 9.5 ± 0.33% respectively, in TXB_2 production (Fig. 2-4). Thus, when estrogen is able to bind to an estrogen receptor it suppresses TXA_2 and PGI_2 production, whereas when this interaction is inhibited, estradiol enhances product formation.

2.3.3. Protein Expression

To help determine the mechanism through which estradiol reduced product formation, enzyme production was measured via Western immunoblot. Interestingly, neither PGHS-1 nor PGHS-2 protein levels were affected in the cells that were incubated with estradiol (Fig. 2-5 A & B). As well, eNOS production was similar in the control and estradiol treated groups (Fig. 2-5 C).

2.4. Discussion

We demonstrated that physiological levels of estradiol suppressed production of the PGHS-dependent eicosanoids, TXA_2 and PGI_2, in endothelial cells. Since production of both TXA_2 and PGI_2 are reduced by estradiol, the inhibition likely occurs at a level preceding the specific synthases. These results are in agreement with the functional data that we have previously reported; where estrogen replacement in ovariectomized rats reduces endothelium-derived PGHS-dependent vasoconstriction.[7] Increased vessel tone as a consequence of vasoconstrictive eicosanoids is common in many pathological conditions such as oxidative stress,[11] hypertension[12] and aging.[6] Thus, in addition to confirming functional data, the present study further elucidates a mechanism by which estradiol attenuates the influence of eicosanoids on the vascular system.

The net effect of estradiol on the eicosanoid pathway is expected to be beneficial to vascular function despite the fact that a known vasodilator, PGI_2, is being suppressed. The reduction of vasoconstrictive eicosanoids is likely of greater efficacy since blocking the TXA_2/PGH_2 receptor has been found to eliminate the enhanced vasoconstriction that occurs in aging,[6] ovariectomized[7] and spontaneously hypertensive[13] rats compared to control rats. In addition, Ermert *et al.* reported that the vasoconstrictive potency of TXA_2 exceeds the vasodilatory capacity of PGI_2.[14] This is especially significant since functional studies with vessels from pathological conditions have shown that nonspecifically blocking the production of eicosanoids with PGHS inhibitors restores vessel tone similar to that of control condition vessels.[13, 15, 16] Studies such as these that have improved vessel function by suppressing the production of all eicosanoids rather

than only vasoconstrictors, further support the hypothesis that estradiol is beneficial by blocking PGHS activity.

Many of the functional studies, including our own, have suggested that the primary PGHS-dependent vasoconstrictor in pathological conditions such as hypertension and oxidative stress is PGH_2 rather than TXA_2.[6, 17] This is particularly significant to our findings since estradiol appears to have suppressed enzyme activity upstream of the specific synthases, possibly at the level of PGHS, which determines production of PGH_2. Hence, our cell culture data of decreased TXA_2 and PGI_2 production suggests that less PGH_2 is available for vasoconstriction.

Tamoxifen, which competitively binds to estrogen receptors,[18] significantly reversed the inhibitory effects of estradiol; indicating that estrogen is likely working through an estrogen receptor-dependent mechanism. The time course data also provides support for the proposed estrogen receptor-dependent effects we observed. When eicosanoid production was measured after 4 or 8 hours of incubation with estradiol, there was no significant change from that of the control condition lacking estradiol. However, after 24 hours of estradiol exposure there was a significant reduction in production of TXA_2 and PGI_2. Interestingly, it appears that a low threshold of estrogen is required to suppress eicosanoid production since our lowest dose exerted maximal inhibition. The fact that TXA_2 production increases when both tamoxifen and estradiol are present, suggests that an opposing non-ER-dependent effect of estradiol is potentially unmasked by tamoxifen.

Considering the tamoxifen experiments indicate that estradiol is affecting the prostaglandin pathway via estrogen receptors, it is feasible that estradiol's mechanism of

action is genomic. However, it was not at the level of altering PGHS expression, as indicated by Western blot analysis. Alternatively, the genomic effects of estradiol may act through other pathways that may ultimately affect PGHS activity. For example, part of estrogen's antioxidant effect is through regulating antioxidant pathways such as superoxide dismutase,[19] glutathione metabolism[20] and glutathione peroxidase activity.[21] Thus, because reactive oxidative species are capable of increasing PGHS activity[22, 23] and inducing PGHS-dependent vasoconstriction,[11] it is plausible that estradiol suppressed eicosanoid production by augmenting antioxidant systems.

Some studies have found that estradiol increases the production of PGI_2 as well as NO,[24-26] whereas our data indicate estradiol decreases eicosanoid production and has no effect on NO. The discrepancy between some of the present findings and previous studies could be due to many factors. For example, the characteristics and function of different vascular beds are heterogeneous.[27,28] Furthermore, the concentration of estradiol must be considered since it can increase prostaglandin production when applied in pharmacological doses.[26] It is important to note, however, that the effect of estradiol on the NO pathway in this experiment is consistent with the suppression of eicosanoid production. NO has previously been found to increase PGHS activity and therefore increase eicosanoid formation.[26,22,29] According to this theory, an increase in NO concentration would have opposed the apparent reduction in PGHS activity in the presence of estradiol. Therefore, it is not surprising that NO production remained at basal levels when estradiol was suppressing eicosanoid synthesis.

In summary, the current study indicates estradiol acts through a receptor dependent process that requires a significant period of time (24 hours) to suppress PGHS activity and thereby reduce eicosanoid synthesis. This novel action of estradiol further elucidates the protective effects of estradiol on the vascular system.

Table 2-1. Specificity of the TXB_2 enzyme immunoassay kit.

Compound/ Specificity		Compound/ Specificity	
Thromboxane B_2	100%	Prostaglandin E_2	0.01%
2,3-dinor Thromboxane B_2	8.2%	6-keto Prostaglandin $F_{1\alpha}$	<0.01%
Prostaglandin D_2	0.44%	2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$	<0.01%
Prostaglandin $F_{2\alpha}$	0.22%	13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$	<0.01%
11-dehydro Thromboxane B_2	0.2%	Leukotriene B_4	<0.01%
Prostaglandin $F_{1\alpha}$	0.05%		

Table 2-2. Specificity of 6-keto Prostaglandin $F_{1\alpha}$ enzyme immunoassay kit.

Compound/ Specificity		Specificity	
6-keto Prostaglandin $F_{1\alpha}$	100%	6,15-diketo-13,14-dihydro prostaglandin $F_{1\alpha}$	2%
Prostaglandin $F_{1\alpha}$	11%	Prostaglandin $F_{2\alpha}$	0.4%
2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$	5%	Thromboxane B_2	0.05%
13,14-dihydro-15-keto Prostaglandin $F_{1\alpha}$	3%	Prostaglandin D_2	<0.01%
Prostaglandin E_2	2%		

Figure 2-1 Effect of 17β-estradiol on thromboxane B_2 production in bovine coronary microvascular endothelial cells. Thromboxane B_2 production was measured from the media of cells in the absence and presence of estradiol [4hrs. (Panel A); 8hrs. (Panel B); and 24 hrs. (Panel C)]. Production is expressed as a percent relative to control values. Each bar represents the mean ± SEM of four individual experiments containing three replicates per condition. *$P<0.05$ vs. control.

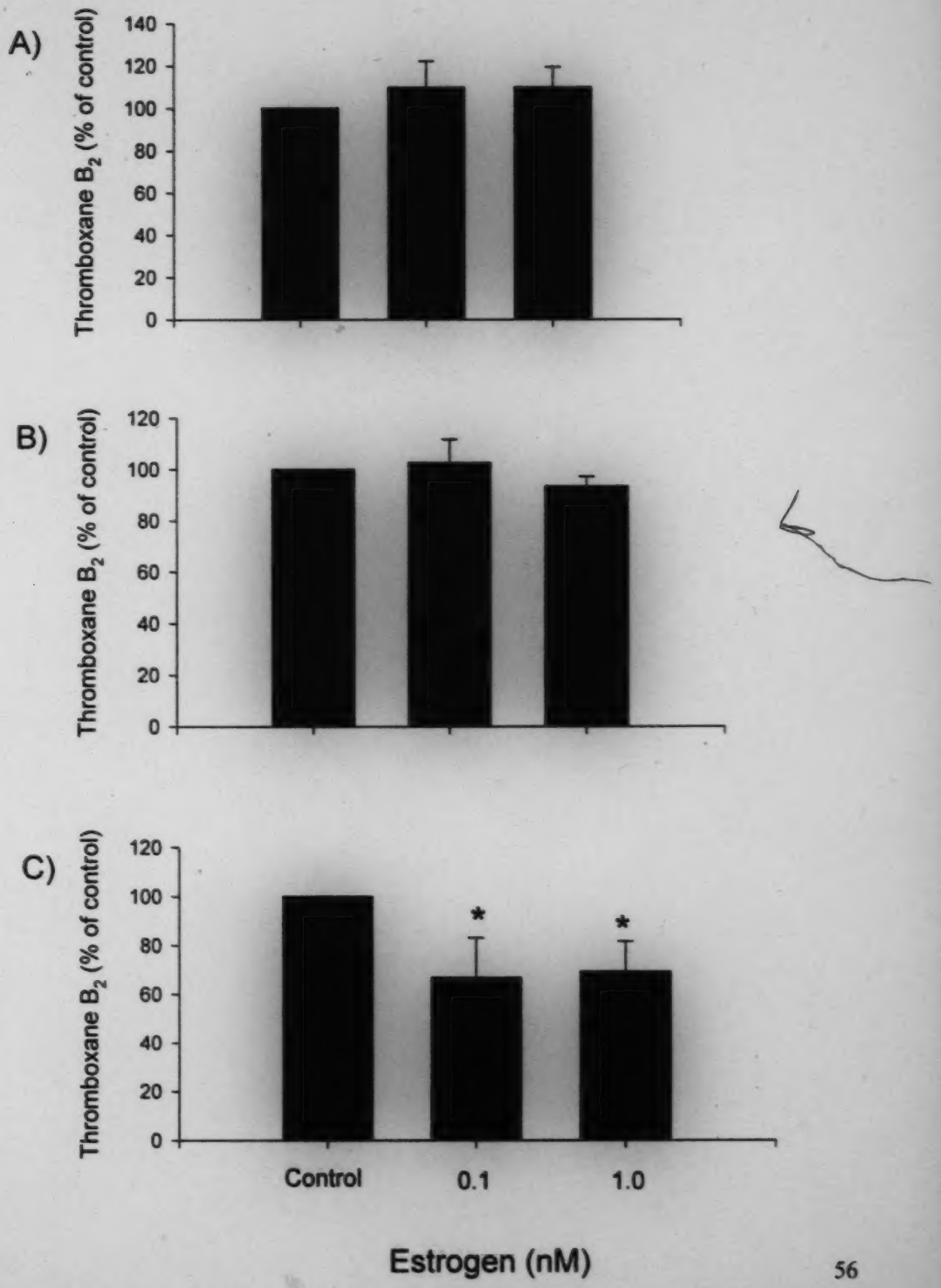

A)
Thromboxane B_2 (% of control)
140
120
100
80
60
40
20
0
B)
Thromboxane B_2 (% of control)
120
100
80
60
40
20
0
C)
Thromboxane B_2 (% of control)
120
100
80
60
40
20
0
*
*
Control
0.1
1.0
Estrogen (nM)

Figure 2-2 6-keto prostaglandin $F_{1\alpha}$ production from bovine coronary microvascular endothelial cells in the absence or presence of estradiol was measured in the media after 24 hours. The data is expressed as the percentage of production relative to the control. Each bar represents the mean ± SEM of three individual experiments containing three replicates per condition. *P<0.05 vs. control.

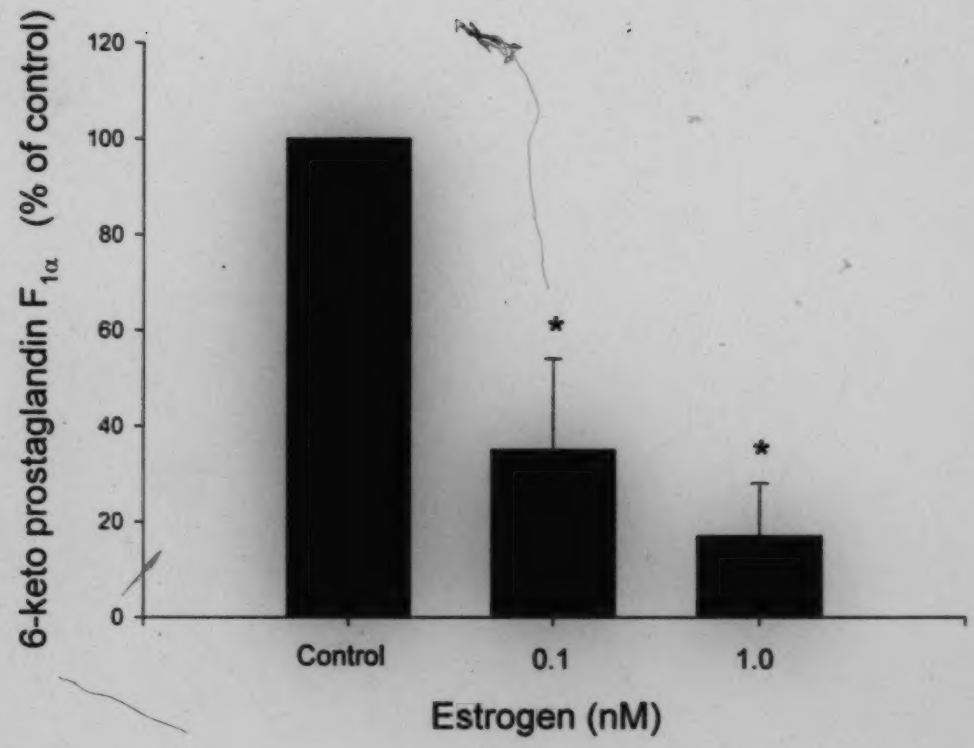

6-keto prostaglandin $F_{1\alpha}$ (% of control)
120
100
80
60
40
20
0
*
*
Control
0.1
1.0
Estrogen (nM)

Figure 2-3 Nitrite production from bovine coronary microvascular endothelial cells in the absence or presence of estradiol was measured in the media. The data is expressed as the percentage of production relative to the control. Each bar represents the mean ± SEM of three individual experiments containing three replicates per condition. There were no significant differences among the groups.

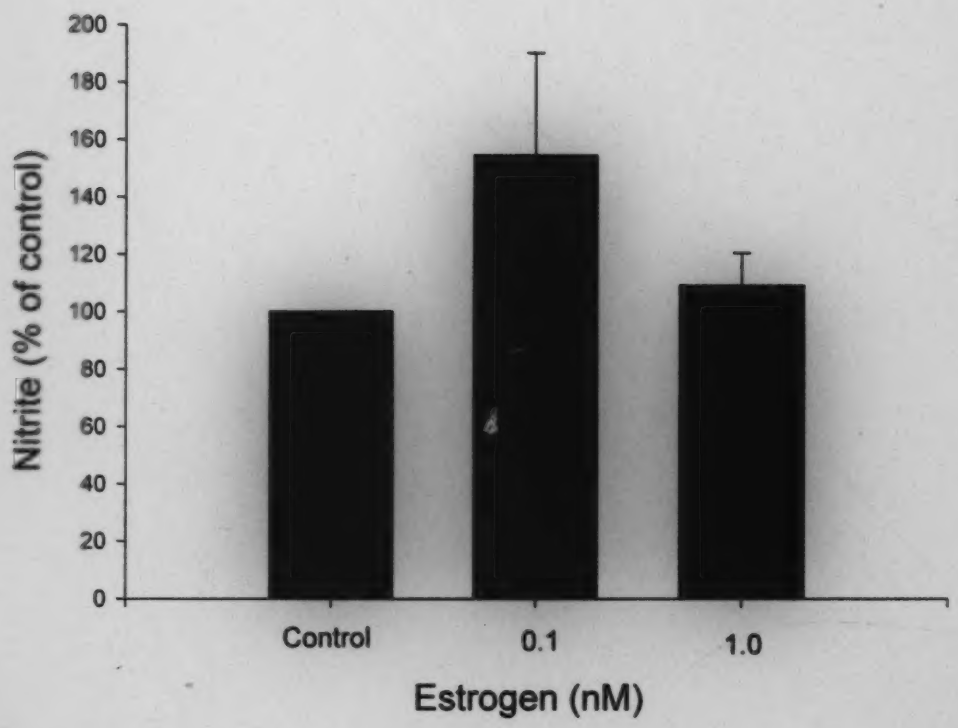

Nitrite (% of control)
200
180
160
140
120
100
80
60
40
20
0
Control
0.1
1.0
Estrogen (nM)

Figure 2-4 Effect of tamoxifen (10^{-7}M) on thromboxane B_2 production in the absence or presence of log doses of estradiol. Compared to tamoxifen alone, estradiol plus tamoxifen caused a significant increase in thromboxane B_2 in the media of bovine coronary microvascular endothelial cells. The data is expressed as the percentage of production relative to the control. Each bar represents the mean ± SEM of three individual experiments containing three replicates per condition. *$P < 0.05$ vs. control.

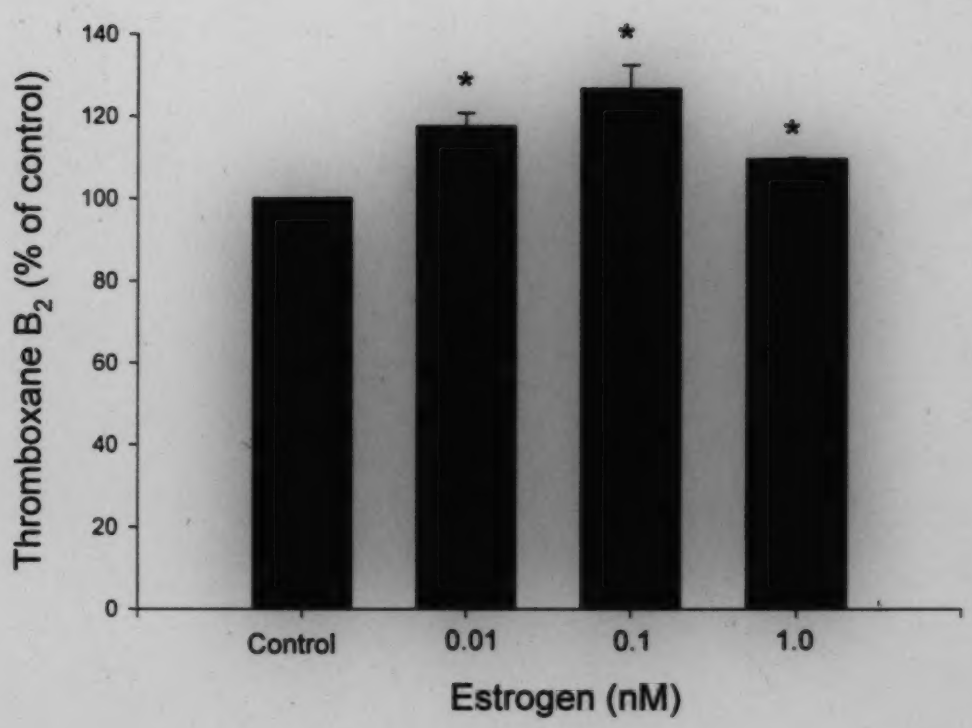
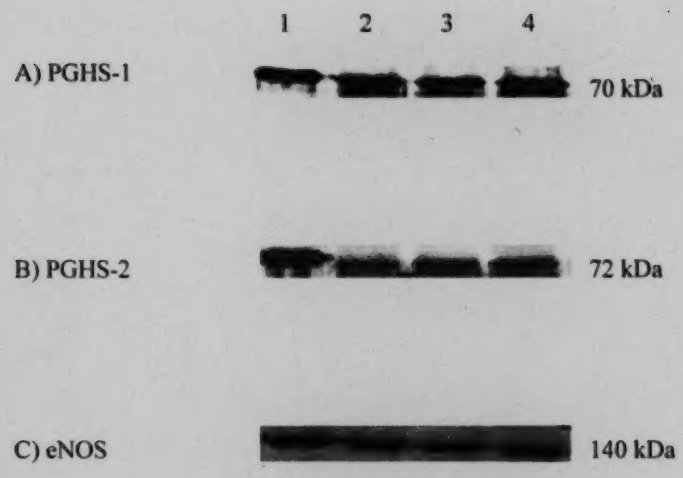

Thromboxane B_2 (% of control)
140
120
100
80
60
40
20
0
*
*
*
Control
0.01
0.1
1.0
Estrogen (nM)

Figure 2-5 Representative Western immunoblots for PGHS-1, PGHS-2, and eNOS in endothelial cells stimulated with 17β-estradiol. Lane 1- standard; Lane 2- untreated cells; Lane 3- 0.1nM estradiol; Lane 4- 1.0nM estradiol. Standards for Panels A, B and C are ovine PGHS-1, ovine PGHS-2, and human endothelial cells, respectively.


1
2
3
4
A) PGHS-1
70 kDa
B) PGHS-2
72 kDa
C) eNOS
140 kDa

2.6. REFERENCES

1. Wenger NK. Cardiovascular disease in the elderly. *Curr Probl Cardiol*.1992;17:609-90.
2. Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, Bender JR. 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization. *Circ Res*.1997;81:885-92.
3. Weiner CP, Lizasoain I, Baylis SA, Knowles RG, Charles IG, Moncada S. Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci U S A*.1994;91:5212-6.
4. Kauser K, Rubanyi GM. Gender difference in endothelial dysfunction in the aorta of spontaneously hypertensive rats. *Hypertension*.1995;25:517-23.
5. Kato T, Iwama Y, Okumura K, Hashimoto H, Ito T, Satake T. Prostaglandin H_2 may be the endothelium-derived contracting factor released by acetylcholine in the aorta of the rat. *Hypertension*.1990;15:475-81.
6. Davidge ST, Hubel CA, McLaughlin MK. Impairment of vascular function is associated with an age-related increase of lipid peroxidation in rats. *Am J Physiol*.1996;271:R1625-31.
7. Davidge S, Zhang Y. Estrogen replacement suppresses a prostaglandin H synthase-dependent vasoconstrictor in rat mesenteric arteries. *Circ. Res*.1998;83:388-395.

8. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*.1976;72:248-54.

9. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem*.1982;126:131-8.

10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*.1970;227:680-5.

11. Rodriguez-Martinez MA, Garcia-Cohen EC, Baena AB, Gonzalez R, Salaices M, Marin J. Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanism involved. *Br J Pharmacol*.1998;125:1329-35.

12. Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y, Fujishima M. Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat. *Hypertension*.1989;14:542-8.

13. Zerrouk A, Auguet M, Chabrier PE. Augmented endothelium-dependent contraction to angiotensin II in the SHR aorta: role of an inducible cyclooxygenase metabolite. *J Cardiovasc Pharmacol*.1998;31:525-33.

14. Ermert L, Ermert M, Althoff A, Merkle M, Grimminger F, Seeger W. Vasoregulatory prostanoid generation proceeds via cyclooxygenase-2 in noninflamed rat lungs. *J Pharmacol Exp Ther*.1998;286:1309-14.

15. Ge T, Hughes H, Junquero DC, Wu KK, Vanhoutte PM, Boulanger CM. Endothelium-dependent contractions are associated with both augmented

expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H_2 in the SHR aorta. *Circ Res*.1995;76:1003-10.

16. Davidge ST, Hubel CA, McLaughlin MK. Cyclooxygenase-dependent vasoconstrictor alters vascular function in the vitamin E-deprived rat. *Circ Res*.1993;73:79-88.

17. Luscher TF, Vanhoutte PM. Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension*.1986;8:344-8.

18. Jordan VC. Biochemical pharmacology of antiestrogen action. *Pharmacol Rev*.1984;36:245-76.

19. Ghanam K, Javellaud J, Ea-Kim L, Oudart N. The protective effect of 17 beta-estradiol on vasomotor responses of aorta from cholesterol-fed rabbit is reduced by inhibitors of superoxide dismutase and catalase. *Biochem Biophys Res Commun*.1998;249:858-64.

20. Kim YD, Farhat MY, Myers AK, Kouretas P, DeGroot KW, Pacquing A, Ramwell PW, Suyderhoud JP, Lees DE. 17-Beta estradiol regulation of myocardial glutathione and its role in protection against myocardial stunning in dogs. *J Cardiovasc Pharmacol*.1998;32:457-65.

21. Massafra C, De Felice C, Gioia D, Buonocore G. Variations in erythrocyte antioxidant glutathione peroxidase activity during the menstrual cycle. *Clin Endocrinol (Oxf)*.1998;49:63-7.

22. Davidge ST, Baker PN, Laughlin MK, Roberts JM. Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. *Circ Res*.1995;77:274-83.

23. Tetsuka T, Daphna-Iken D, Miller BW, Guan Z, Baier LD, Morrison AR. Nitric oxide amplifies interleukin 1-induced cyclooxygenase-2 expression in rat mesangial cells. *J Clin Invest*.1996;97:2051-6.

24. Hayashi T, Fukuto JM, Ignarro LJ, Chaudhuri G. Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: implications for atherosclerosis. *Proc Natl Acad Sci U S A*.1992;89:11259-63.

25. Cicinelli E, Ignarro LJ, Lograno M, Galantino P, Balzano G, Schonauer LM. Circulating levels of nitric oxide in fertile women in relation to the menstrual cycle. *Fertil Steril*.1996;66:1036-8.

26. Myers SI, Turnage RH, Bartula L, Kalley B, Meng Y. Estrogen increases male rat aortic endothelial cell (RAEC) PGI_2 release. *Prostaglandins Leukot Essent Fatty Acids*.1996;54:403-9.

27. Thorin E, Shatos MA, Shreeve SM, Walters CL, Bevan JA. Human vascular endothelium heterogeneity. A comparative study of cerebral and peripheral cultured vascular endothelial cells. *Stroke*.1997;28:375-81.

28. Chen L, McNeill JR, Wilson TW, Gopalakrishnan V. Heterogeneity in vascular smooth muscle responsiveness to angiotensin II. Role of endothelin. *Hypertension*.1995;26:83-8.

29. Salvemini D, Seibert K, Masferrer JL, Misko TP, Currie MG, Needleman P. Endogenous nitric oxide enhances prostaglandin production in a model of renal inflammation. *J Clin Invest*.1994;93:1940-7.

3. AGING INCREASES PGHS-2 DEPENDENT VASOCONSTRICTION IN RAT MESENTERIC ARTERIES.

3.1. Introduction

The process of aging is associated with a variety of deleterious adaptations that can have pathological effects on the vasculature. For example, during aging the production of superoxide anions increases[1, 2] while antioxidant systems are suppressed[3-5] and production of the vasodilator, nitric oxide (NO), is decreased.[5] Although decreased endothelium-dependent relaxation[6, 7] is associated with aging, the specific mechanisms for such alterations in vascular responsiveness are not completely understood.

The endothelium contributes to the regulation of vessel tone by releasing vasodilators and vasoconstrictors[8] that modulate both physiological and pathophysiological processes. The nitric oxide synthase (NOS) and prostaglandin H synthase (PGHS) pathways are of particular importance as they have a substantial influence on vessel function and are affected by the processes of aging.[7, 9-12] Eicosanoids produced from the PGHS pathway induce vasodilation and vasoconstriction; whereas NO generally decreases vessel tone.

The enzyme PGHS constitutes a rate limiting step in the synthesis of eicosanoids. It exists in at least two isoforms, PGHS-1 and PGHS-2, and is responsible for converting the initial substrate, arachidonic acid, into the pathway intermediate PGH_2. In addition to functioning as a potent vasoconstrictor, PGH_2 can be further converted by terminal synthases and isomerases into vasoactive compounds such as TXA_2 and PGI_2. Under

normal physiological conditions, the eicosanoid pathway generally induces vasorelaxation. However, in certain conditions such as hypertension[13, 14] and aging[7, 12] there is an imbalance where vasoconstrictors become much more prominent and result in significantly greater vessel tone.

Aging has been associated with an increase in reactive oxidative species (ROS)[1, 2] as well as decreased antioxidant capacity.[3, 4, 15] Both increased ROS production[2] and decreased antioxidant activity[15] contribute to an elevated level of oxidative stress that can have pathological ramifications on the eicosanoid[16-18] and NO[19] pathways. For example, ROS facilitate activation of the transcription factor, NF-KB,[18] which binds to the promoter region of the PGHS-2 gene and therefore upregulates expression of the enzyme.[20, 21] The functional consequence of increased PGHS expression is that it is pathological in many animal models by augmenting vasoconstriction.[12-14] Moreover, ROS further erode the balance between dilators and constrictors by decreasing PGI_2 synthase activity.[22] Finally, the eicosanoid pathway is also responsible for elevating the level of oxidative stress since it is a direct source of ROS production.[23, 24]

The NO pathway is another prominent modulator of vessel function that generally induces vessel relaxation. Except, it too has been found to have pathological effects in conditions such as elevated oxidative stress where superoxide anions react with NO to produce the cytotoxic substance peroxynitrite.[19, 25] Superoxide anions also attenuate endothelium-dependent vasorelaxation by consuming NO and thereby reduce the amount of vasodilator available to act on the vascular smooth muscle.[26] This can lead to enhanced vessel tone and blood pressure. Conversely, by activating inducible nitric

oxide synthase (iNOS),[27] oxidative stress can stimulate the production of excessive and detrimental amounts of NO.[28-30]

We have previously shown aging increases the level of oxidative stress as well as enhances PGHS-dependent vasoconstriction.[12] However, the differential modulation of vessel function by PGHS-1 vs. PGHS-2 has not been determined. Given the age-associated increase in PGHS-2-inducing factors such as ROS[1, 2] and cytokines,[31-33] as well as predominance of PGHS-2 in conditions such as Alzheimer's disease[34] and arthritis,[35] we hypothesized that the PGHS-2 isoform contributes to increased vessel tone in aging. Moreover, determining the effect of PGHS-2 on vascular function in aging could lead to the clinical use of newly developed specific PGHS-2 inhibitors. Therefore, the goal of this study was to determine: 1) changes in vascular function with age 2) how PGHS-dependent modulation of vessel function changes with age 3) how NO-dependent modulation of vascular function changes with age.

3.2. Methods

3.2.1 General Animal Model

Female Sprague-Dawley rats (2 months of age) were obtained from Biological Sciences (University of Alberta, Canada) and housed in the facilities at the University of Alberta. The rats were aged to either 3 months (n=9) or 12 months (n=14). On the day of experiment, they were killed under light anesthesia with methohexital sodium (50mg/kg body wt). The animal protocols were examined by the University of Alberta Animal Welfare Committee and found to be in compliance with the guide-lines issued by the Canada Council on Animal Care.

3.2.2 Experimental Design

Mesenteric arteries averaging 250 μm in diameter were mounted in an isometric myograph system. Four separate baths were used to study arterial segments simultaneously. Cumulative doses of phenylephrine (1 μmol/L to 50 μmol/L) were administered to measure sensitivity and determine the dose that would give a 50 % contraction for each individual artery. The EC_{50} of phenylephrine was used to constrict arteries in order to achieve a baseline from which subsequent relaxation responses were measured. After completion of each dose-response curve, a 30-minute recovery period was allowed during which the baths were changed every 10 minutes with fresh HEPES-buffered physiological saline solution (sodium chloride 142, potassium chloride 4.7, magnesium sulfate 1.17, calcium chloride 1.56, potassium phosphate 1.18, HEPES 10, and glucose 5.5 mmol/L, pH 7.4). The studies performed involving the absence and presence of inhibitors were conducted sequentially on the same artery. Vessels were preincubated with inhibitors for 15 minutes prior to methacholine dose response curves (1 nmol/L to 1 μmol/L).

Methacholine relaxation curves were generated in the absence or presence of the NO synthase inhibitor, N^G –monomethyl-L-arginine (L-NMMA, 250 μmol/L), to determine a role for NO in the responses. To verify the pharmacological inhibition of the NOS pathway, dose response curves to L-NMMA (3 μmol/L to 30 mmol/L) were performed in arteries preconstricted to 10% of their maximum response to phenylephrine. Sequential doses of L-NMMA were then applied to the vessels and the level of constriction in response to each dose of inhibitor was recorded. Maximum vessel constriction was reached at concentrations of L-NMMA below that used experimentally;

suggesting that NOS activity was optimally inhibited. As well, the effect of the PGHS inhibitor, meclofenamate (10 μmol/L); PGHS-1 inhibitor, valeryl salicylate (3 mmol/L); PGHS-2 inhibitor, NS-398 (10 μmol/L) and TXA_2/PGH_2 receptor antagonist SQ-29548 (1 μmol/L) on methacholine-induced relaxation were measured.

Vascular smooth muscle sensitivity to the exogenous NO donor sodium nitroprusside (SNP) (1nmol/L - 1μmol/L), TXA_2 mimetic U-46619 (1nmol/L – 0.1μmol/L) and phenylephrine were also measured. For these dose response curves, arteries from the same rats were denuded of endothelium. Endothelium removal was done mechanically by threading a human hair through the lumen of the artery.[36] Confirmation of complete endothelium removal was assessed pharmacologically with a single dose of 1 μmol/L methacholine.

The reproducibility of repeating curves for these experiments was determined in a preliminary set of experiments designed to test for tachyphylaxis.

As previously described,[37] western immunoblotting was performed for PGHS-1 and PGHS-2 with primary monoclonal antibodies (mouse anti-PGHS-1 and anti-PGHS-2; Cayman Chemical Co., Ann Arbor, MI, USA).

Phenylephrine, methacholine, meclofenamate and sodium nitropursside were all purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Valeryl salicylate, NS-398 and SQ-29548 were all purchased from Cayman Chemical Company (Ann Arbor, MI, USA). L-NMMA was purchased from Calbiochem-Novabiochem International (San Diego, CA, USA).

3.2.3. Data Analysis

The data from the dose-response curves were fitted to the Hill equation, from which a straight line was generated by linear least-squares regression analysis. The EC_{50} was determined from this line and expressed as the geometric means +/- SE. Comparison between groups was done by a two-way analysis of variance. Post hoc analysis for comparison between groups was performed using a Tukey test. Differences among means were considered significant at $P < 0.05$.

Western immunoblot bands from young and aged vessels were quantified by densitometry and then analyzed with a student's t-test. Differences among means were considered significant at $P < 0.05$.

3.3. Results

3.3.1. Vascular Responses

There was no significant difference in response to phenylephrine (endothelium intact or denuded) between the two groups (Fig. 3-1).

Mesenteric arteries preconstricted with their EC_{50} of phenylephrine demonstrated a concentration-dependent relaxation response to methacholine in the young and aged groups. Although both groups relaxed to 100%, the aging group had a substantially blunted relaxation response to methacholine; as indicated by a larger EC_{50} value (Fig. 3-2).

PGHS inhibitors were used to determine the role of the prostaglandin pathway with respect to the altered vascular responses in the aging group. The PGHS inhibitor, meclofenamate, significantly enhanced relaxation in the aging group but had no effect on

the young (Fig. 3-3A). In the presence of meclofenamate there was no difference between the two groups. To determine which isoform was predominantly responsible for the augmented vasoconstriction in the aging group, specific PGHS-1 and PGHS-2 inhibitors were used.[38] Valeryl salicylate, a PGHS-1 inhibitor, did not affect vessel relaxation in either group (Fig. 3-3B). Conversely, the PGHS-2 inhibitor, NS-398, greatly enhanced relaxation in the aging group and restored vessel function to that of the young group; which was not influenced by the inhibitor (Fig. 3-3C). To elucidate the identity of the vasoconstrictive eicosanoid(s) in the aging group, the TXA_2/PGH_2 receptor antagonist, SQ-29548, was applied to the vessel baths. Similar to PGHS inhibition, the receptor blocker did not affect relaxation in the young group (Fig. 3-3D). However, inhibiting the receptor in the aging group enhanced relaxation in a manner similar to PGHS-2 inhibition (Fig. 3-3D).

The role of NO in methacholine-induced relaxation was determined by inhibiting the NOS pathway with L-NMMA. Inhibiting NO production in the young group significantly blunted the relaxation response although did not prevent vessels from reaching maximal relaxation (Fig. 3-4). In contrast, NOS inhibition did not affect relaxation to methacholine in the aging group (Fig. 3-4). Complete inhibition of NOS activity was verified by a dose response curve to L-NMMA reaching a vasoconstriction plateau at drug concentrations below that used in the experiments (data not shown).

A TXA_2 receptor agonist and exogenous NO donor were applied to endothelium denuded vessels to determine whether or not smooth muscle sensitivity was partially responsible for the differential responses of vessels in the two groups. There was no difference in sensitivity or maximum responses between the young and aging vessels

when a dose response curve to the thromboxane mimetic U-46619 was measured (Fig. 3-5). As well, endothelium denuded vessels in the two groups responded similarly to SNP (Fig. 3-6).

3.3.2. Western Immunoblot

To provide insight into the cellular mechanisms determining vessel function, protein expression was measured in mesenteric arteries. As expected based on the functional data, there was no significant difference in PGHS-1 protein expression in the young and aged group (arbitrary units = 339 ± 74 vs. 429 ± 14). In agreement with the functional results, PGHS-2 protein expression in the mesenteric arteries was significantly higher in the aging group compared to the young group (Fig. 3-7).

3.4. Discussion

The purpose of this study was to further characterize the changes in vascular function that occur during aging. Although we previously demonstrated that PGHS-dependent vasoconstriction is increased in rat mesenteric vessels during aging,[12] it has not previously been determined whether PGHS-1 or PGHS-2 is the primary isoform responsible for the increase in vasoconstriction. In the present study, we found that PGHS-2 greatly contributes to vasoconstriction in mesenteric arteries during aging, whereas PGHS-1 does not have a significant effect on methacholine-induced relaxation in the young or aging group. Collectively inhibiting both PGHS isoforms or selectively inhibiting PGHS-2, resulted in the aging vessels functioning similar to young vessels under control conditions (without an inhibitor). Furthermore, blocking the TXA_2/PGH_2

receptor also eliminated the blunted relaxation in the aging vessels. Thus, modulation of vessel function via the TXA_2/PGH_2 receptor becomes much more dominant in aging as a result of increased PGHS-2 activity. This is very significant with the advent of specific PGHS-2 inhibitors that could potentially reverse the blunted relaxation associated with aging.

In contrast with the blunted relaxation response to methacholine in the aged group, there was no significant difference in vasoconstriction to phenylephrine between the two groups. Furthermore, removing the endothelium did not affect the response to phenylephrine in either group. Thus, basal release of vasoactive compounds by the endothelium and smooth muscle sensitivity to phenylephrine were not changed in our model of aging. It appears that stimulation of the endothelium by a vasorelaxant such as methacholine is necessary to induce the release of PGHS-2-dependent vasoconstrictors in the aged group.

Functional differences between young and aging vessels could be a consequence of both endothelial cell product formation and vascular smooth muscle sensitivity. However, our data indicate that the functional changes were the result of altered endothelial metabolism rather than changes in smooth muscle responsiveness. For example, there was no difference in smooth muscle sensitivity to exogenous NO, as SNP induced similar relaxation in both groups. Hence, the smooth muscle in vessels from young and aging rats is equally sensitive to NO-induced relaxation. Moreover, the thromboxane mimetic U-46619 induced vessel constriction to a similar degree in young and aging vessels. Therefore, the enhanced vessel tone associated with the aging group is not due to increased PGH_2/TXA_2 receptor responsiveness.

It is also interesting to note that in the mesenteric vascular bed, the eicosanoid pathway does not appear to have a vasodilatory role when stimulated with methacholine. If significant amounts of PGI_2 were present in mesenteric arteries of young rats, relaxation would have been suppressed when PGHS was inhibited. However, inhibiting PGHS activity in young rats had no effect on relaxation. Konishi *et al.* also observed a lack of PGHS-dependent vasorelaxation in rat mesenteric arteries.[39]

The present study clearly demonstrates the pathologic influence of eicosanoids on vascular function during aging. Western immunoblot data suggests that increased PGHS-2 protein mass in mesenteric arteries contributes to the enhanced vasoconstriction in aging; whereas, PGHS-1 protein mass did not change with age. There are a variety of potential mechanisms for the augmented role of PGHS-2-dependent vasoconstriction.[20, 40-42] Specific to aging, cytokines become more prominent with age[31-33] and are capable of increasing PGHS-2 expression.[43-45] Oxidative stress could also lead to an upregulation of PGHS-2 protein expression via NF-kB activation.[20, 40]

As well, ROS are capable of increasing vessel tone by reducing the activity of prostacyclin synthase.[22] Although PGHS is considered to be the rate limiting step in the eicosanoid pathway,[46] it has been suggested by Camacho *et al.* that attenuated prostacyclin synthase activity could result in excess intermediate (PGH_2) being available to directly act on the vasculature or to be shunted toward other terminal enzymes such as thromboxane synthase.[22]

Finally, it is possible that aging is associated with stimuli that favor the synthesis of vasoconstrictive eicosanoids. Indeed, the coupling of a specific phospholipase(s), PGHS isoform, and terminal synthase(s) can be favored depending on the stimuli.[47]

Thus, during aging undetermined factors may favor the production of vasconstrictive eicosanoids by stimulating the segregated functional coupling of phospholipases with PGHS-2 and possibly thromboxane synthase. Furthermore, Murakami *et al.* has demonstrated the ability of PGHS-2, but not PGHS-1, to initiate the release of endogenous arachidonic acid.[48] Therefore, in addition to catalyzing the rate-limiting step in the eicosanoid pathway, increased PGHS-2 expression may further enhance vessel tone by augmenting substrate availability and consequently facilitating the production of vasoconstrictors.

As well, we found the role of NO in methacholine-induced relaxation decreases with age. When the NOS inhibitor, L-NMMA, was added to the vessel baths relaxation was significantly blunted in the young group; suggesting that NO plays a dominant role in relaxing these vessels. Conversely, NOS inhibition did not affect relaxation to methacholine in the aging group. Thus, other factors appear to be responsible for relaxing microvessels in aging. Indeed, endothelium derived hyperpolarizing factor (EDHF) has been shown to be a dominant regulator of relaxation in microvessels.[49, 50] However, past studies have reported that EDHF is more prominent in young than aged rats.[51]

Our observation of decreased NO-mediated relaxation in aging is in agreement with Cernadas *et al.* and Chou *et al.* who recently found that aging decreases the activity of eNOS in the aorta.[10, 11] One of the potential causes of reduced NOS activity is an age-related decrease in cofactors.[52] As well, the attenuation of NO-mediated relaxation in the aging group is likely, in part, a result of free radicals reacting with NO; thereby consuming available relaxing factor and forming cytotoxic compounds such as peroxynitrite.[53] This hypothesis is supported by the facts that aging is associated with

elevated levels of ROS[1, 2] and decreased levels of SOD expression[3] and activity.[5] Interestingly, the proposed scavenging of NO and formation of peroxynitrite is also in accordance with our functional data regarding the eicosanoid pathway. After all, peroxynitrite has been found to increase PGHS activity[54] yet inhibit prostacyclin synthase activity.[55]

In conclusion, we found that aging is associated with increased PGHS-2 dependent vasoconstriction. Furthermore, NO-dependent modulation of vessel tone is virtually eliminated in aging. Thus, we have identified PGHS-2 as the predominant isoform in aging and therefore suggest that inhibition of this isoform is potentially a new target for therapeutic intervention to improve vascular function.

Figure 3-1 EC_{50} values for phenylephrine in vessels with intact endothelium (solid bars) and without endothelium (open bars) in mesenteric arteries from young (n=11, n=4, respectively) and aged (n= 8, n=5 respectively) rats. Bars represent mean ± SEM (no significant difference).

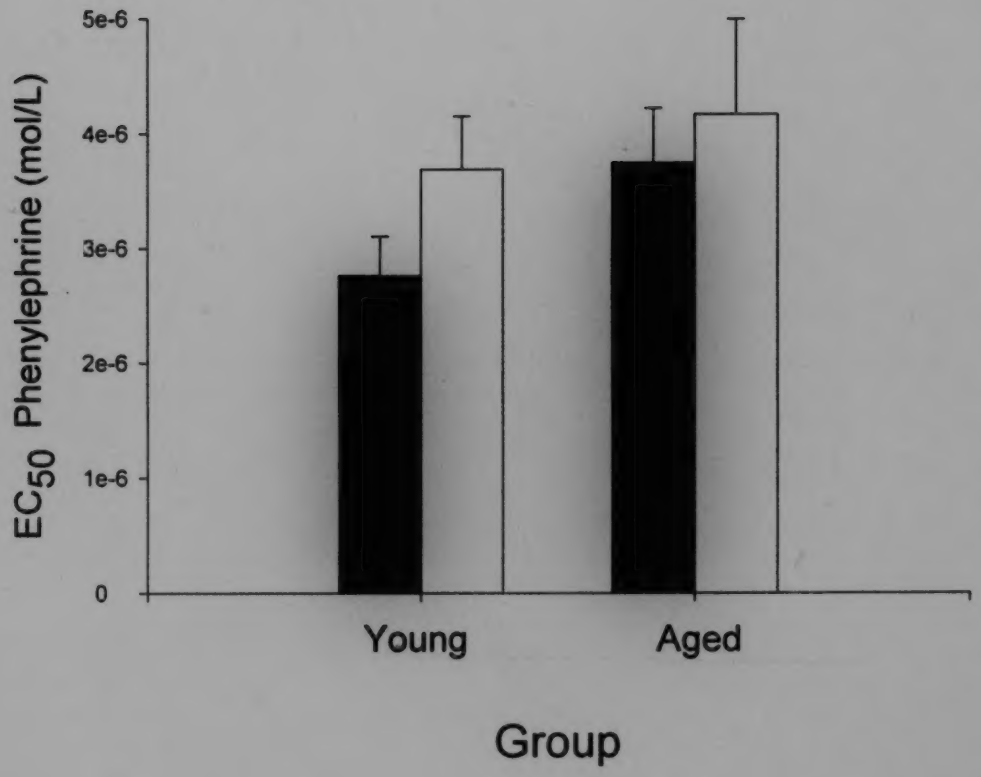

5e-6
4e-6
3e-6
2e-6
1e-6
0
EC50 Phenylephrine (mol/L)
Young
Aged
Group

Figure 3-2 Concentration response curves to methacholine in young (n=9, •) and aged (n=8, o) rats. Responses are expressed as a percent of maximum relaxation. The inset graph represents EC_{50} values for methacholine induced relaxation. #P<0.05 vs young.

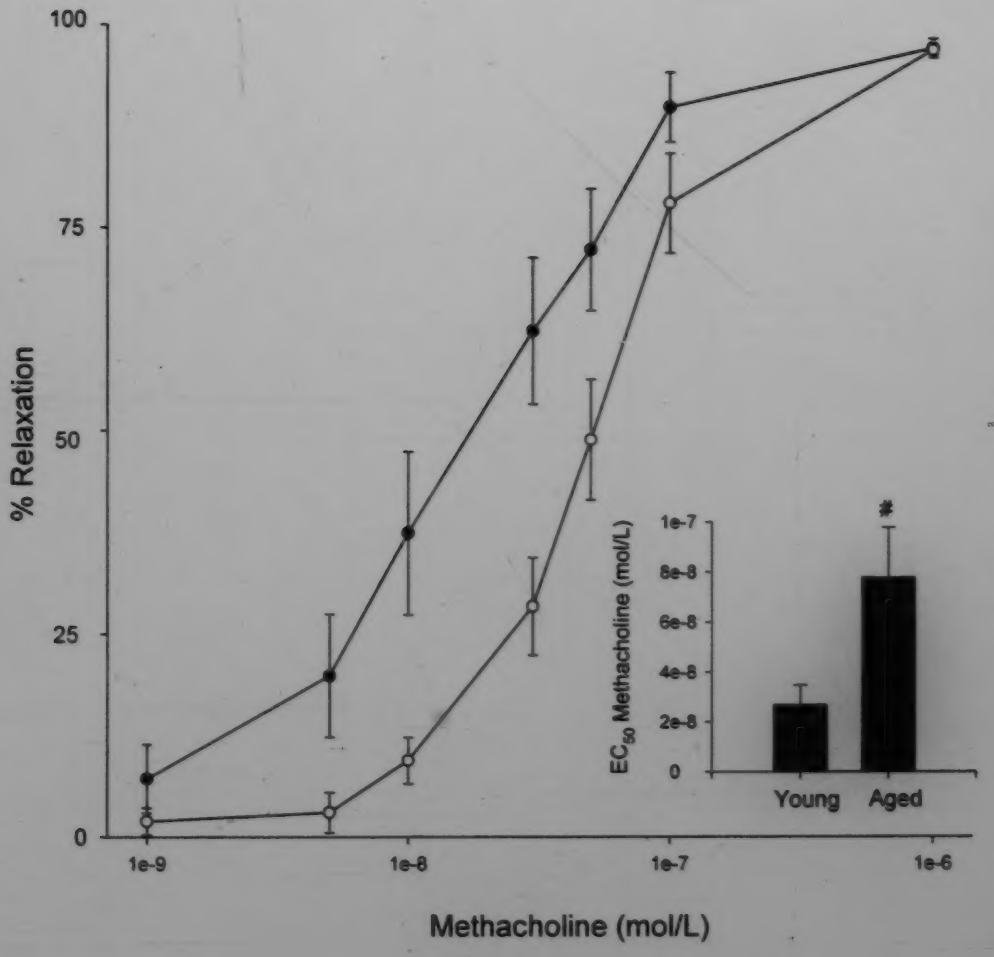

100
75
50
25
0
% Relaxation
1e-9
1e-8
1e-7
1e-6
Methacholine (mol/L)
EC50 Methacholine (mol/L)
1e-7
8e-8
6e-8
4e-8
2e-8
0
#
Young
Aged

Figure 3-3 Bar graphs showing EC_{50} values for relaxation to methacholine alone (solid bars) and methacholine in the presence of PGHS pathway inhibitors (open bars) in mesenteric arteries from young (3 months) and aged (12 months) rats. A) Meclofenamate- an inhibitor of both PGHS isoforms (young: n=9; aged: n=8); B) Valeryl salicylate- a PGHS-1 inhibitor (young: n=9; aged: n=8); C) NS-398- a PGHS-2 inhibitor (young: n= 7; aged: n=8); and D) SQ-29548- a TXA_2/PGH_2 receptor blocker (young:n=6; aged: n=8). Bars represent mean ± SEM. *P<0.05 vs methacholine alone, #P<0.05 vs young.

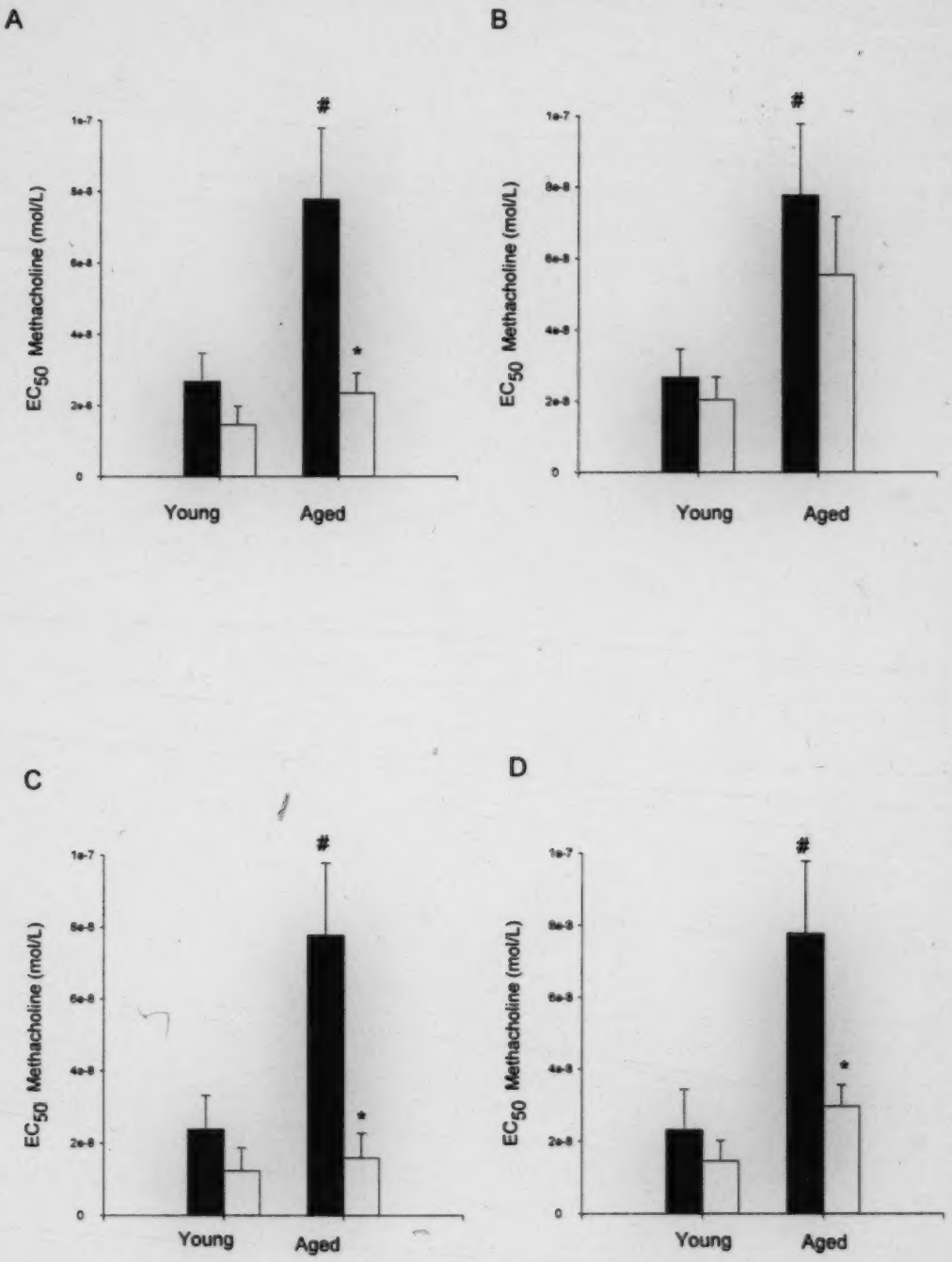

A
B
C
D
EC_{50} Methacholine (mol/L)
#
*
Young
Aged

Figure 3-4 EC_{50} values for relaxation to methacholine alone (solid bars) and methacholine in the presence of L-NMMA, a NOS inhibitor, (open bars) in mesenteric arteries from young (n=9) and aged (n=6) rats. Bars represent mean ± SEM. *P<0.05 vs methacholine alone, #P<0.05 vs young.

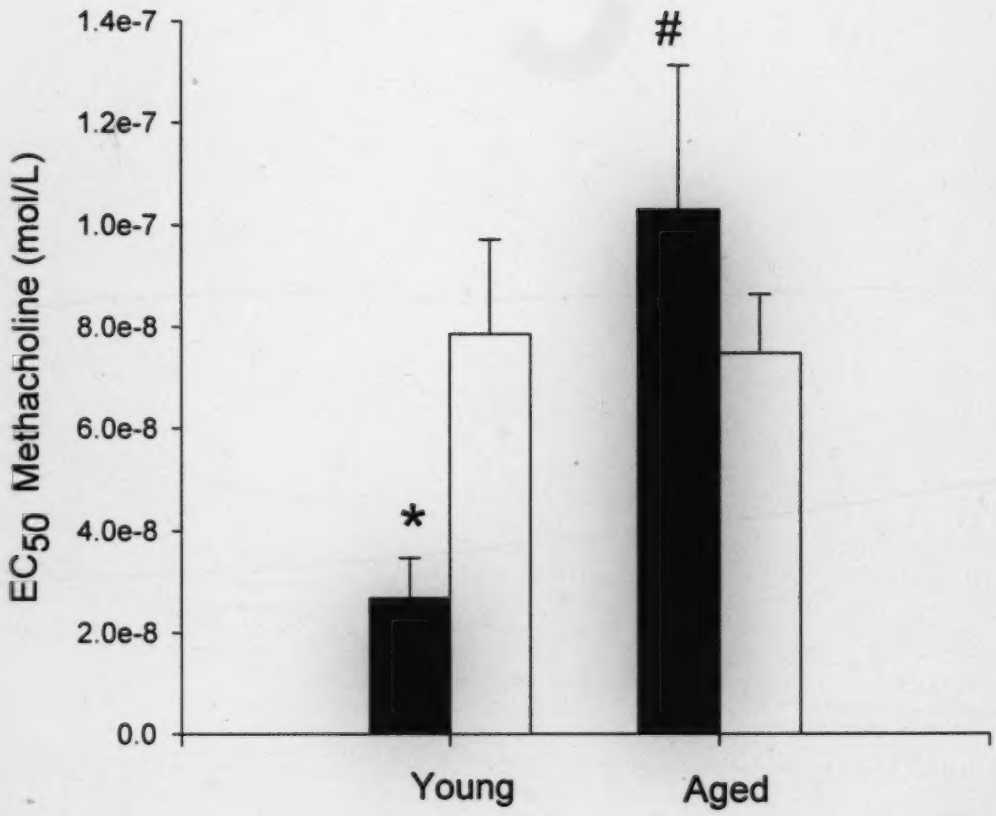

1.4e-7
1.2e-7
1.0e-7
8.0e-8
6.0e-8
4.0e-8
2.0e-8
0.0
EC50 Methacholine (mol/L)
*
#
Young
Aged

Figure 3-5 Concentration-response curves to U-46619 in mesenteric arteries from young (n= 4, •) and aged (n=7, o) rats. Responses are expressed as a percentage of maximum constriction to the TXA_2 mimetic, U-46619. Data represent mean ± SEM (no significant difference).

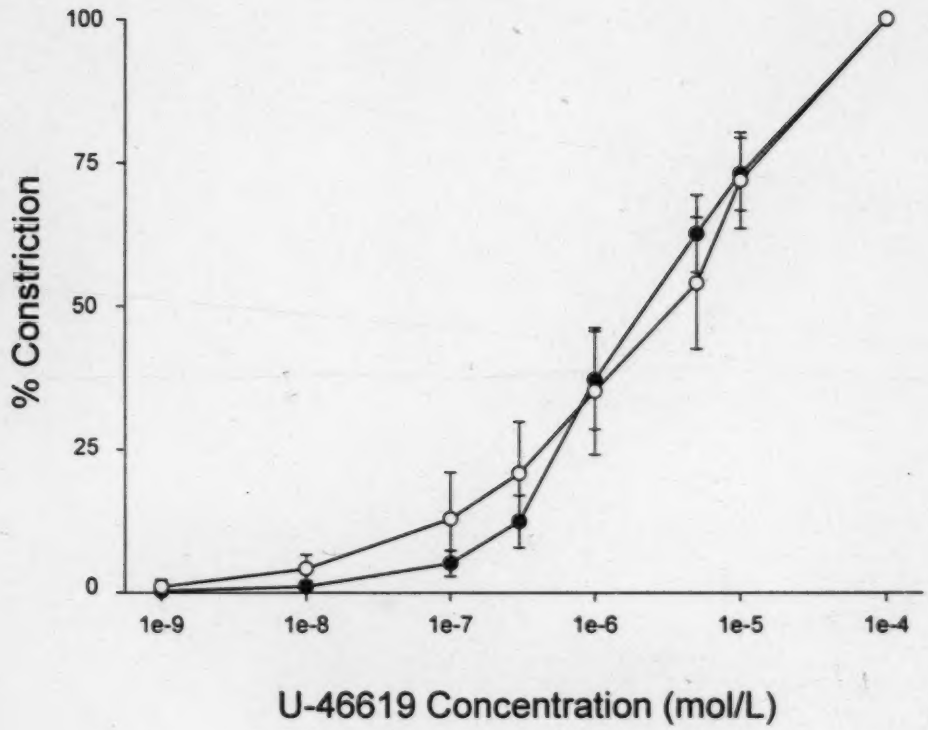

100
75
50
25
0
% Constriction
1e-9
1e-8
1e-7
1e-6
1e-5
1e-4
U-46619 Concentration (mol/L)

Figure 3-6 Concentration-response curves to SNP in mesenteric arteries from young (n= 5, •) and aged (n=8, o) rats. Responses are expressed as percent relaxation. Data represent mean ± SEM (no significant difference).

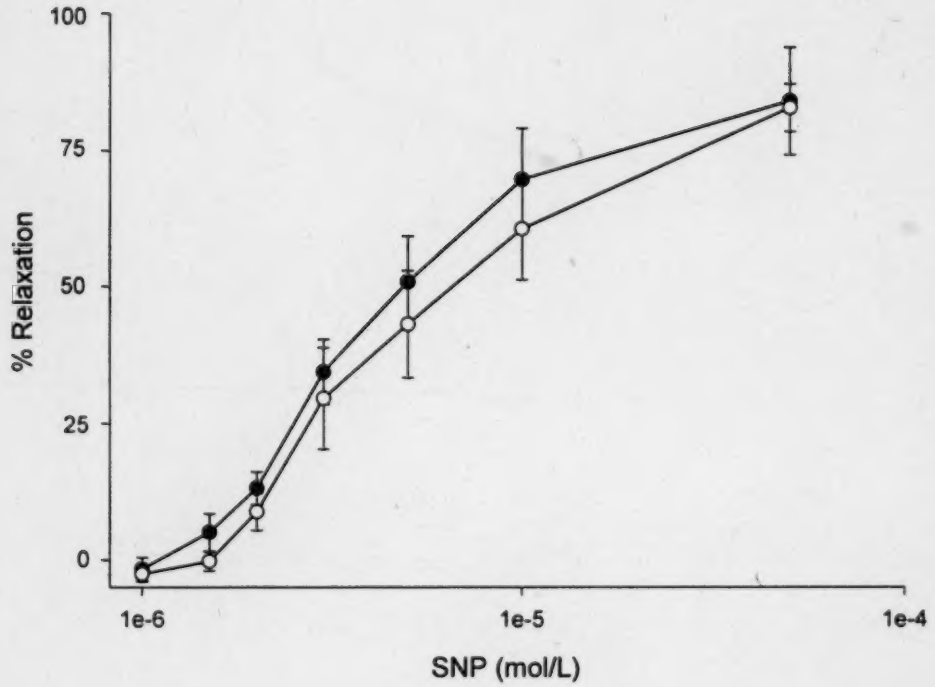
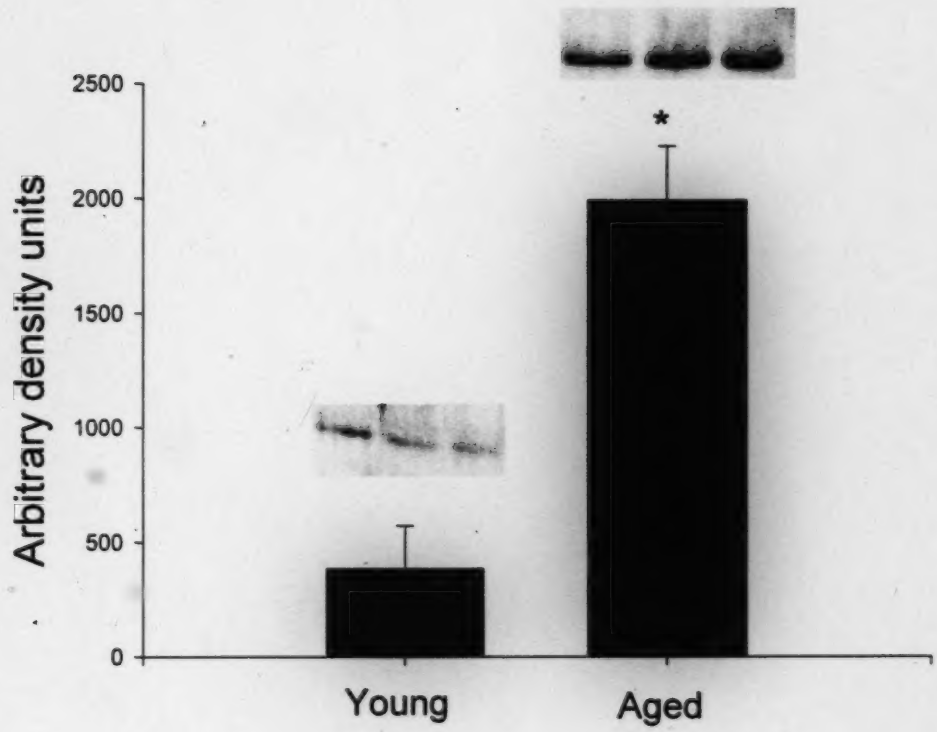

100
75
50
25
0
% Relaxation
1e-6
1e-5
1e-4
SNP (mol/L)

Figure 3-7 Arbitrary densitometry values to quantify representative Western immunoblot bands for PGHS-2 protein expression in mesenteric arteries from young and aged rat vessels. Each bar represents the mean ± SEM of three samples. *P < 0.05 vs. young, student's t-test.


2500
2000
1500
1000
500
0
Arbitrary density units
*
Young
Aged

3.5. REFERENCES

1. Nohl H, Hegner D. Do mitochondria produce oxygen radicals in vivo? *Eur J Biochem*.1978;82:563-7.
2. Sohal RS, Ku HH, Agarwal S, Forster MJ, Lal H. Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mech Ageing Dev*.1994;74:121-33.
3. Azhar S, Cao L, Reaven E. Alteration of the adrenal antioxidant defense system during aging in rats. *J Clin Invest*.1995;96:1414-24.
4. Xia E, Rao G, Van Remmen H, Heydari AR, Richardson A. Activities of antioxidant enzymes in various tissues of male Fischer 344 rats are altered by food restriction. *J Nutr*.1995;125:195-201.
5. Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S, Luscher TF. Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. *Hypertension*.1997;30:817-24.
6. Moritoki H, Hosoki E, Ishida Y. Age-related decrease in endothelium-dependent dilator response to histamine in rat mesenteric artery. *Eur J Pharmacol*.1986;126:61-7.
7. Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y, Fujishima M. Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat. *Hypertension*.1989;14:542-8.
8. Luscher TF. The endothelium. Target and promoter of hypertension? *Hypertension*.1990;15:482-5.

9. Challah M, Nadaud S, Philippe M, Battle T, Soubrier F, Corman B, Michel JB. Circulating and cellular markers of endothelial dysfunction with aging in rats. *Am J Physiol*.1997;273:H1941-8.

10. Cernadas MR, Sanchez de Miguel L, Garcia-Duran M, Gonzalez-Fernandez F, Millas I, Monton M, Rodrigo J, Rico L, Fernandez P, de Frutos T, Rodriguez-Feo JA, Guerra J, Caramelo C, Casado S, Lopez F. Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. *Circ Res*.1998;83:279-86.

11. Chou TC, Yen MH, Li CY, Ding YA. Alterations of nitric oxide synthase expression with aging and hypertension in rats. *Hypertension*.1998;31:643-8.

12. Davidge ST, Hubel CA, McLaughlin MK. Impairment of vascular function is associated with an age-related increase of lipid peroxidation in rats. *Am J Physiol*.1996;271:R1625-31.

13. Ge T, Hughes H, Junquero DC, Wu KK, Vanhoutte PM, Boulanger CM. Endothelium-dependent contractions are associated with both augmented expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H_2 in the SHR aorta. *Circ Res*.1995;76:1003-10.

14. Kato T, Iwama Y, Okumura K, Hashimoto H, Ito T, Satake T. Prostaglandin H_2 may be the endothelium-derived contracting factor released by acetylcholine in the aorta of the rat. *Hypertension*.1990;15:475-81.

15. Tian L, Cai Q, Wei H. Alterations of antioxidant enzymes and oxidative damage to macromolecules in different organs of rats during aging. *Free Radic Biol Med*.1998;24:1477-84.

16. Roberts LJ, 2nd, Montine TJ, Markesbery WR, Tapper AR, Hardy P, Chemtob S, Dettbarn WD, Morrow JD. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J Biol Chem*.1998;273:13605-12.

17. Ji YS, Xu Q, Schmedtje JF, Jr. Hypoxia induces high-mobility-group protein I(Y) and transcription of the cyclooxygenase-2 gene in human vascular endothelium. *Circ Res*.1998;83:295-304.

18. Speir E, Shibutani T, Yu ZX, Ferrans V, Epstein SE. Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection. *Circ Res*.1996;79:1143-52.

19. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*.1996;271:C1424-37.

20. Lukiw WJ, Bazan NG. Strong nuclear factor-kappaB-DNA binding parallels cyclooxygenase-2 gene transcription in aging and in sporadic Alzheimer's disease superior temporal lobe neocortex. *J Neurosci Res*.1998;53:583-92.

21. Schmedtje JF, Jr., Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem*.1997;272:601-8.

22. Camacho M, Lopez-Belmonte J, Vila L. Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity. *Circ Res*.1998;83:353-65.

23. Tsai AL, Palmer G, Kulmacz RJ. Prostaglandin H synthase. Kinetics of tyrosyl radical formation and of cyclooxygenase catalysis. *J Biol Chem*.1992;267:17753-

9.

24. Lassmann G, Odenwaller R, Curtis JF, DeGray JA, Mason RP, Marnett LJ, Eling TE. Electron spin resonance investigation of tyrosyl radicals of prostaglandin H synthase. Relation to enzyme catalysis [published erratum appears in J Biol Chem 1992 Mar 25;267(9):6449]. *J Biol Chem.*1991;266:20045-55.

25. Brunelli L, Crow JP, Beckman JS. The comparative toxicity of nitric oxide and peroxynitrite to Escherichia coli. *Arch Biochem Biophys.*1995;316:327-34.

26. Davidge ST, Ojimba J, McLaughlin MK. Vascular function in the vitamin E-deprived rat: an interaction between nitric oxide and superoxide anions. *Hypertension.*1998;31:830-5.

27. Adcock IM, Brown CR, Kwon O, Barnes PJ. Oxidative stress induces NF kappa B DNA binding and inducible NOS mRNA in human epithelial cells. *Biochem Biophys Res Commun.*1994;199:1518-24.

28. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med.*1993;329:2002-12.

29. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J.*1992;6:3051-64.

30. Palmer RM, Bridge L, Foxwell NA, Moncada S. The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br J Pharmacol.*1992;105:11-2.

31. Belmin J, Bernard C, Corman B, Merval R, Esposito B, Tedgui A. Increased production of tumor necrosis factor and interleukin-6 by arterial wall of aged rats. *Am J Physiol.*1995;268:H2288-93.

32. Prechel MM, Halbur L, Devata S, Vaidya AM, Young MR. Increased interleukin-6 production by cerebral cortical tissue of adult versus young mice. *Mech Ageing Dev*.1996;92:185-94.

33. Ye SM, Johnson RW. Increased interleukin-6 expression by microglia from brain of aged mice. *J Neuroimmunol*.1999;93:139-48.

34. Ho L, Pieroni C, Winger D, Purohit DP, Aisen PS, Pasinetti GM. Regional distribution of cyclooxygenase-2 in the hippocampal formation in Alzheimer's disease. *J Neurosci Res*.1999;57:295-303.

35. Geis GS. Update on clinical developments with celecoxib, a new specific COX-2 inhibitor: what can we expect? *Scand J Rheumatol Suppl*.1999;109:31-7.

36. Osol G, Cipolla M, Knutson S. A new method for mechanically denuding the endothelium of small (50-150 microns) arteries with a human hair. *Blood Vessels*.1989;26:320-4.

37. Davidge ST, Hubel CA, McLaughlin MK. Cyclooxygenase-dependent vasoconstrictor alters vascular function in the vitamin E-deprived rat. *Circ Res*.1993;73:79-88.

38. Johnson JL, Wimsatt J, Buckel SD, Dyer RD, Maddipati KR. Purification and characterization of prostaglandin H synthase-2 from sheep placental cotyledons. *Arch Biochem Biophys*.1995;324:26-34.

39. Konishi C, Naito Y, Saito Y, Ohara N, Ono H. Age-related differences and roles of endothelial nitric oxide and prostanoids in angiotensin II responses of isolated, perfused mesenteric arteries and veins of rats. *Eur J Pharmacol*.1997;320:175-81.

40. Speir E, Yu ZX, Ferrans VJ, Huang ES, Epstein SE. Aspirin attenuates

cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells. *Circ Res*.1998;83:210-6.

41. Peri KG, Varma DR, Chemtob S. Stimulation of prostaglandin G/H synthase-2 expression by arachidonic acid monoxygenase product, 14,15-epoxyeicosatrienoic acid. *FEBS Lett*.1997;416:269-72.

42. Wu KK, Hatzakis H, Lo SS, Seong DC, Sanduja SK, Tai HH. Stimulation of de novo synthesis of prostaglandin G/H synthase in human endothelial cells by phorbol ester. *J Biol Chem*.1988;263:19043-7.

43. Nakagawa T, Fujita N, Oh-Hara T, Kurokawa T, Nakamura K, Tsuruo T. Interleukin-1 alpha induced cyclooxygenase-2 expression in bone-derived endothelial cells. *J Cell Physiol*.1999;179:226-32.

44. Vlahos R, Stewart AG. Interleukin-1alpha and tumour necrosis factor-alpha modulate airway smooth muscle DNA synthesis by induction of cyclo-oxygenase-2: inhibition by dexamethasone and fluticasone propionate. *Br J Pharmacol*.1999;126:1315-24.

45. Bany BM, Kennedy TG. Role of interleukin 1 in the regulation of cyclooxygenase gene expression in rat endometrial stromal cells. *J Reprod Fertil*.1999;115:125-31.

46. Smith WL, Marnett LJ, DeWitt DL. Prostaglandin and thromboxane biosynthesis. *Pharmacol Ther*.1991;49:153-79.

47. Naraba H, Murakami M, Matsumoto H, Shimbara S, Ueno A, Kudo I, Oh-ishi S. Segregated coupling of phospholipases A_2, cyclooxygenases, and terminal prostanoid synthases in different phases of prostanoid biosynthesis in rat

peritoneal macrophages. *J Immunol*.1998;160:2974-82.

48. Murakami M, Kambe T, Shimbara S, Kudo I. Functional coupling between various phospholipase A_2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *J Biol Chem*.1999;274:3103-15.

49. Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A. Importance of endothelium-derived hyperpolarizing factor in human arteries [published erratum appears in J Clin Invest 1998 Mar 15;101(6):1491]. *J Clin Invest*.1997;100:2793-9.

50. de Wit C, Esser N, Lehr HA, Bolz SS, Pohl U. Pentobarbital-sensitive EDHF comediates ACh-induced arteriolar dilation in the hamster microcirculation. *Am J Physiol*.1999;276:H1527-34.

51. Fujii K, Ohmori S, Tominaga M, Abe I, Takata Y, Ohya Y, Kobayashi K, Fujishima M. Age-related changes in endothelium-dependent hyperpolarization in the rat mesenteric artery. *Am J Physiol*.1993;265:H509-16.

52. Strolin Benedetti M, Dostert P, Marrari P, Cini M. Effect of ageing on tissue levels of amino acids involved in the nitric oxide pathway in rat brain. *J Neural Transm Gen Sect*.1993;94:21-30.

53. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A*.1990;87:1620-4.

54. Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci U S A*.1996;93:15069-74.

55. Zou M, Martin C, Ullrich V. Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem*.1997;378:707-13.

4. DISCUSSION

4.1. Summary

It has been established that aging is associated with blunted vasorelaxation responses to endothelium-dependent agonists.[1-5] Eicosanoids are recognized as important modulators of vessel function that are affected by a variety of physiological states.[6-9] However, the role of eicosanoids in aging has not been extensively studied. As well, estrogen greatly decreases the incidence of cardiovascular disease in aging women; although the majority of its mechanisms of action have not been determined. Thus the objectives of my thesis were to further elucidate the effects of estrogen and aging on the PGHS and NOS pathways in the vascular endothelium. In particular, we attempted to: 1) Examine how estrogen affects production of eicosanoids and NO in cultured vascular endothelial cells. 2) Determine how aging affects PGHS and NOS-dependent regulation of vascular function. 3) Measure the effect of estrogen-replacement on the PGHS and NOS pathways, with respect to vessel function in aging.

Two experimental models were used to address the questions posed. To examine the PGHS and NOS pathways at the level of cellular processes, vascular endothelial cells were exposed to physiological levels of estrogen. To compliment this model, we examined the functional effects of estrogen and aging by measuring vessel function in young, aged, and estrogen-replaced aged rats. However, technical difficulties, explained below in more detail, prevented us from addressing the effects of estrogen on vascular function in aging.

Based on our past finding of decreased PGHS-dependent vasoconstriction in estrogen-replaced ovariectomized rats compared to control condition ovariectomized

rats,[10] we concluded that estrogen reduces the efficacy of vasoconstrictive eicosanoids. Thus, we hypothesized that physiological levels of estrogen would reduce production of TXA_2 and possibly other products, in cultured endothelial cells. Because estrogen has been found to increase NO production in previous studies,[11, 12] we also expected an increase in NO production in our cell culture model. Our hypotheses of decreased vasoconstrictive eicosanoid and increased NO synthesis represent two of the potential mechanisms whereby estrogen could be protective to the vasculature.

Our first study showed that estrogen:

1) Reduced the production of TXA_2 and PGI_2 after 24 hours of exposure.
2) Did not significantly affect eicosanoid synthesis when exposed to cells for only 4 or 8 hours.
3) Did not inhibit eicosanoid production in the presence of tamoxifen, an estrogen receptor antagonist.
4) Had no effect on NO production.
5) Did not affect PGHS-1, PGHS-2, or eNOS protein expression.

From this study, we concluded that estrogen likely suppressed eicosanoid production by inhibiting PGHS activity via estrogen receptors.

Both our previous data[7] and other studies[6] indicate that aging is associated with enhanced PGHS-dependent vasoconstriction. Given the dominant role of PGHS-2 in conditions such as arthritis[13] and Alzheimer's Disease,[14] we expected that vascular function in aging may also be influenced by the inducible isoform. We hypothesized that

PGHS-2-dependent vasoconstrictors would blunt endothelium-dependent relaxation in aging.

The following results were observed (Figure IV-1.):

1) The sensitivity and maximum response of vessels to phenylephrine were not endothelium-dependent nor was there an effect of age.
2) Relaxation to methacholine was significantly blunted in the aged group.
3) In the aged group, inhibition of either PGHS-2 or the PGH_2/TXA_2 receptor reversed the blunted relaxation response and restored vessel function to that of the young group.
4) Conversely, PGHS-1 inhibition did not affect relaxation in either the young or aged group.
5) Vascular smooth muscle sensitivity to the TXA_2 mimetic, U-46619, and exogenous NO-donor, SNP, was not changed with age.
6) Protein expression of PGHS-1 did not change with age, whereas both PGHS-2 and eNOS expression increased significantly in the aged group.

Because we observed a significant age-associated increase in PGHS-2-dependent vasoconstriction, we suggest that the recently developed specific PGHS-2 inhibitors may be effective in reducing the incidence of cardiovascular disease in aging.

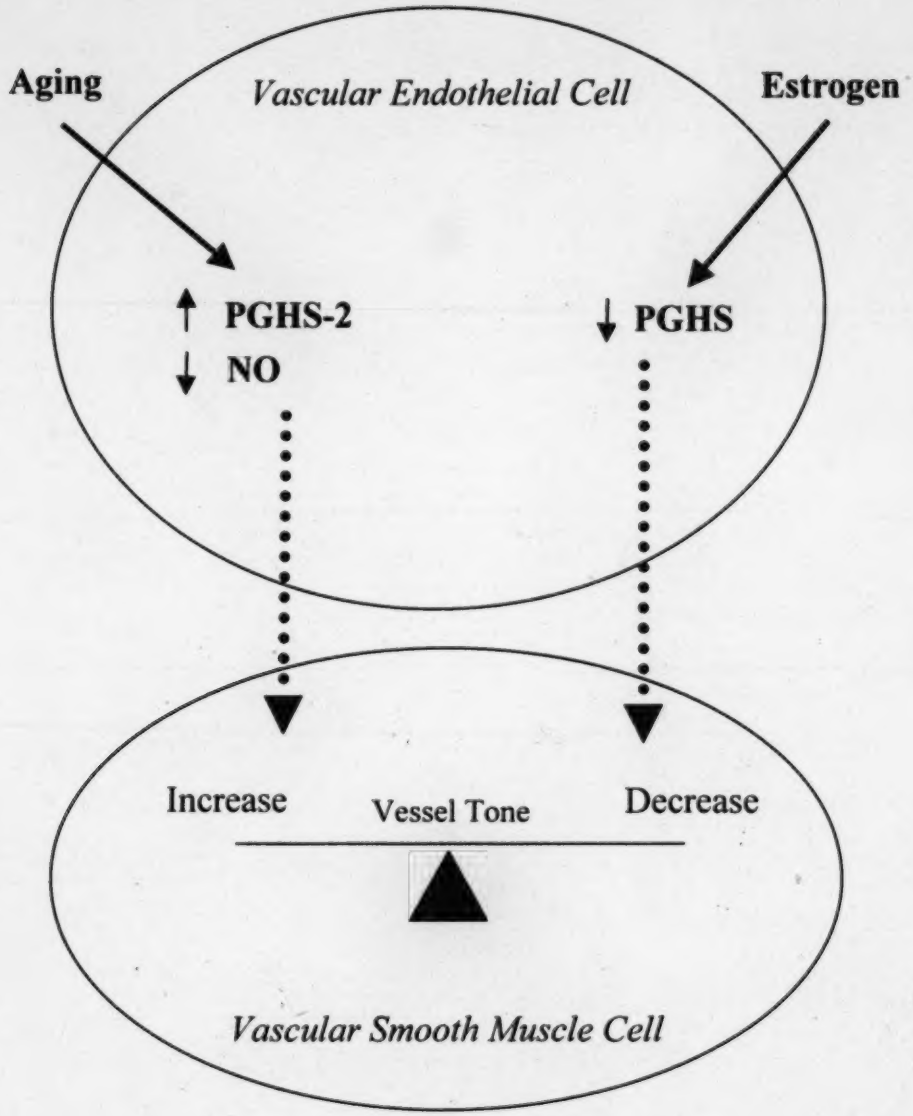


Figure 4-1. Summary of thesis results.

4.2. Speculation

The fact that estrogen suppressed synthesis of both PGI_2 and TXA_2 in our cultured endothelial cells, suggests that estrogen was acting upstream of the specific terminal enzymes. PGHS and phospholipases are two of the potential targets for estrogen to inhibit. Specifically, estrogen may suppress eicosanoid production by decreasing substrate availability through reduced synthesis or activity of phospholipases. It has been found to suppress oxidant-induced release of arachidonic acid by phospholipase A_2.[15] In addition, Graber *et al.* reported a delayed but sustained inhibitory effect of estrogen on phospholipase C activity.[16] However, several previous studies assessing the effect of estrogen on phospholipases demonstrated estrogen increasing enzyme activity in an ER-dependent manner.[17-20] Thus, estrogen may not suppress eicosanoid production through altered phospholipid hydrolysis.

It is also interesting to note that Cabot *et al.* found that tamoxifen increases phospholipase activity.[21] Such an effect could explain the increased TXA_2 formation we observed when both estradiol and tamoxifen were applied to the cells. Thus, unopposed estrogen suppressed eicosanoid production in our cell culture model, through an unidentified mechanism such as PGHS-inhibition. However, in the presence of tamoxifen, phospholipase activity could have been enhanced; while the inhibitory effect of estrogen was blocked. The net result of such an effect would be increased eicosanoid production as we observed.

Alternatively, estrogen may have suppressed eicosanoid production in the cultured endothelial cells by inhibiting PGHS. Modulation of PGHS activity is a complex process that is affected by a number of factors. The regulation of hydroperoxide

levels is a likely mechanism through which estrogen attenuates PGHS activity. After all, hydroperoxide is responsible for activating PGHS,[22] and estrogen enhances the effect of glutathione peroxidase, an enzyme responsible for scavenging hydroperoxide.[23, 24] Thus, estrogen might reduce the catalytic activity of PGHS by decreasing the amount of hydroperoxide available to activate the enzyme. Similarly, by enhancing the effect of SOD[25] estrogen could potentially reduce the concentration of superoxide anions and consequently attenuate peroxynitrite formation, which is also known to activate PGHS.[26] Finally, estrogen is known to have a variety of other antioxidant effects,[25, 27] which ultimately could decrease activation of PGHS.

PGHS activity can also be influenced at the level of tyrosine phosphorylation. Facilitating tyrosine phosphorylation with protein tyrosine phosphatase inhibitors results in increased PGHS activity in the cerebral vasculature.[28, 29] Similarly, incubating aortic endothelial cells with sodium fluoride, results in a tryrosine phosphorylation-dependent activation of PGHS.[30] It is possible that estrogen posttranslationally attenuates PGHS activity by facilitating the dephosphorylation of PGHS.

Our data suggest that estrogen suppresses eicosanoid synthesis by acting at the level of PGHS which prevents production of both PGH_2 and TXA_2. This mechanism of inhibition is expected to be beneficial despite reducing PGI_2 production, because the vasoconstrictive potency of TXA_2 has been found to exceed the vasodilatory capacity of PGI_2.[31] Furthermore, nonselective inhibition of the PGHS pathway in a variety of pathological conditions restores vessel tone to that of control conditions.[32-34] Hence, estrogen's proposed mechanisms of action in our cell culture study can explain the reduction in vessel tone that we previously observed with estrogen replacement in young

rats.[10] Our cell culture data also supports the hypothesis that part of estrogen's cardioprotective effect in postmenopausal women is through a suppression of vasoconstrictive eicosanoids.

To fully understand the effects of estrogen in postmenopausal women, we must also characterize the influence of aging on vascular function. In our studies, the eicosanoid pathway appears to be the primary factor responsible for the blunted relaxation response to methacholine in aged rats since inhibition of the PGHS-2 pathway restored the function of aged vessels to that of young. As in previous studies[9, 35-37] our data suggests that PGHS-dependent vasoconstriction is derived from the endothelium. In the presence of a PGHS-2 inhibitor, endothelium dependent relaxation was virtually identical in the young and aged groups. As well, endothelium denuded vessels from the young and aged groups responded similarly to both SNP and phenylephrine.

A number of factors could have contributed to the increased PGHS-2-dependent vasoconstriction in aging by manipulating substrate availability as well as PGHS activity and protein mass. For example, aging is associated with elevated levels of ROS that could increase arachidonic acid availability,[38, 39] and enhance PGHS activation.[40, 41] Furthermore, cytokines which increase with age,[42-44] elevate PGHS-2 protein mass.[45-47]

In addition to the factors mentioned above, there are several other stimuli that regulate PGHS-2 expression in complex manner. For example, the cytochrome P450 monoxygenase product, 14, 15-epoxyeicosatrienoic acid, increases PGHS-2 mRNA and protein synthesis.[48] This demonstrates that metabolites of arachidonic acid exert an effect on PGHS expression. Endothelin may have also contributed to PGHS-2 dependent vasoconstriction in aging given that it increases PGHS-2 expression[49-52] and becomes

more predominant with age.[53-55] Although all the stimuli responsible for inducing PGHS-2 expression have not been identified, tyrosine kinase is known to be a common mediator of increased PGHS-2 expression.[49, 50, 56]

The NOS pathway may also contribute to increased PGHS activity in aging. After all, NO and superoxide anions react to form peroxynitrite[57] which is capable of increasing PGHS activity.[26] Thus, considering that NO-mediated relaxation was virtually eliminated in the aging group and eNOS protein mass increased, we recently investigated whether or not peroxynitrite formation was more prominent in aging. Our preliminary immunohistochemistry results indicate that peroxynitrite formation, as evidenced by tyrosine nitrosylation, is significantly greater in the aortic endothelium in aging (unpublished data). This evidence provides another possible mechanism for the increased influence of PGHS-2 on vascular function in aging. Moreover, it also supports our observation of decreased NO-mediated relaxation, as peroxynitrite formation represents the scavenging of biologically active NO.

Our study on vascular function indicates that PGHS-2 is chronically upregulated in aging. Although this observation is in opposition with the dogma that views PGHS-2 as an inducible isoform, there is substantial evidence supporting a sustained role for the enzyme in some circumstances. For example, PGHS-2 is fundamental in the developmental processes of several tissues,[58-60] and is constitutively expressed in certain cell types.[31, 61, 62] The emerging applications of specific PGHS-2 inhibitors in treating chronic conditions such as arthritis,[13] lupus nephritis,[63] and Alzheimer's Disease[14] further substantiate the sustained influence of the enzyme. Thus, it is reasonable to suggest that aging is a state of chronically elevated arterial PGHS-2 expression. As well,

our cell culture study suggests that a portion of estrogen's vasoprotective effect may be through suppressing an age-associated increase in PGHS-2 dependent vasoconstriction.

4.3. Limitations

4.3.1. Animal Model

Although we elucidated an effect of estrogen on the PGHS pathway and also identified PGHS-2 as a novel isoform contributing to blunted relaxation in aging, we were unable to explore all of our areas of interest. Most importantly, our model addressing the effect of age on vessel function was designed with an aged estrogen-replaced group. This group would have enabled us to determine whether or not estrogen suppresses the production of vasoconstrictive eicosanoids in our model of aging. However, the aged Sprague-Dawley rats were much larger (body weight of approximately 500g) than most 12-months old rats reported in the literature and therefore the 0.5mg estradiol pellets were not sufficient to significantly increase estrogen plasma levels. Consequently, we cannot make any conclusions regarding the effect of estrogen replacement on eicosanoids and vascular function in aging.

We expected that estrogen replacement would at least partially reverse the blunted relaxation response in the aged group for a number of reasons. First, previous work has shown that estrogen is capable of directly inducing relaxation by reducing intracellular calcium concentrations[64] and enhancing the efflux of potassium ions.[11] Second, estrogen has been found to increase the release of NO in many studies.[11, 12, 65] Third, by decreasing production of PGI_2 and TXA_2, our cell culture study demonstrated that

estrogen inhibits the PGHS pathway. Finally, we previously showed that estrogen replacement in young ovariectomized rats reduces PGHS-dependent vasconstriction.[10]

4.3.2. Experimental Equipment

Although the vessel myograph system used to measure the force with which vessels constrict is a very effective method to analyze vessel function, the system is not conducive to the measurement of product formation. The vessels reside in baths with a large volume (5 ml) that dilutes the vessel metabolite to below detectable limits for an assay. The ability to measure and compare the amounts of vasoactive compounds produced by each vessel would be a valuable addition to the functional data because it would allow us to determine the profile of vasoactive substances produced as well as the ratio of PGH_2 and TXA_2 released.

4.4. Future Directions

There are various experimental models that could be utilized to further advance our knowledge of the effects of estrogen and aging on vascular function. First, it is important to measure vessel function in aged rats in the absence and presence of estrogen. Increased plasma concentrations of estrogen can readily be achieved by implanting more concentrated estrogen pellets. This study will be a great addition to our previous work because it will determine whether or not estrogen reduces the age-associated PGHS-2-dependent vasoconstriction and reverses the decrease in NO-mediated relaxation.

Examining the effect of chronic PGHS-2 inhibition in aging is another project that would expand on our recent work. Drugs have recently been developed that specifically

inhibit the PGHS-2 isoform without interrupting activity or expression of the constitutive PGHS-1 isoform.[66] This experimental design will achieve at least two valuable goals. First, if chronic inhibition of PGHS-2 restores vessel function to that of young vessels, it would confirm the efficacy of this isoform in aging. Second, it will allow for a limited comparison between the benefits of estrogen replacement versus PGHS-2 inhibition. Our previous data have indicated that the primary benefit of estrogen replacement is suppressing PGHS-dependent vasoconstriction. Yet, if estrogen replacement improves vessel relaxation to a greater extent than PGHS-2 inhibition, we will conclude that estrogen has additional and unidentified effects on vascular function in our model.

Both of these models are very versatile with respect to the questions that can be addressed. Because the PGHS and NOS pathways modulate vessel function in response to numerous agonists, we can determine how estrogen replacement and PGHS-2 inhibition affect PGHS and NOS-mediated regulation of vessel tone in response to stimuli such as bradykinin, angiotensin II, and calcitonin gene related peptide (CGRP). These peptides are examples of vasoactive compounds that could potentially be affected by the process of aging. For example, bradykinin is known to stimulate phospholipases, which in turn provide substrate for eicosanoid production. Therefore, if PGHS-2 becomes more prominent in aging and leads to the production of vasoconstrictors, bradykinin could have significantly different effects in aged vessels compared to young.

Similarly, CGRP-mediated regulation of vascular function may be altered by age and estrogen. We have recently demonstrated that matrix metalloproteinase (MMP)-2 reduces the potency of CGRP by cleaving it into smaller fragments (data not published). As well, aging[67, 68] and estrogen (data not published) have been associated with elevated

expression of MMP protein. Thus, the influence of estrogen and aging on MMPs may ultimately decrease the efficacy of CGRP-mediated relaxation. Considering that the mechanism(s) by which CGRP induces vasodilation is not well known,[69] it is possible that either estrogen replacement or chronic PGHS-2 inhibition could modify the effect of CGRP.

Angiontensin II is another important factor contributing to the regulation of vascular tone. Further delineating the influence of this peptide in models of estrogen-replacement and PGHS-2 inhibition would provide valuable insight; as both estrogen and eicosanoids mediate the effect of angiotensin II on the vasculature. Both chronic[70] and acute[71] exposure to estrogen has been found to attenuate angiotensin II-induced contraction in rat aortic rings. Decreased angiotensin-converting enzyme mRNA expression[72] and activity[73, 74] are two of the mechanisms whereby estrogen reduces the efficacy of angiotensin. The potentially beneficial effects of suppressing angiotensin-converting enzyme include decreased production of a vasoconstrictor as well as reduced metabolism of the vasodilator bradykinin. Similarly, the effect of angiotensin II would likely also be modified in a model of PGHS-2 inhibition. Indeed, angiotensin II induces eicosanoid-mediated vasoconstriction in monkey and dog cerebral arteries,[75] as well as in rat aortic rings.[32] This suggests that PGHS-2 inhibition may also attenuate the vasoconstrictive effect of angiotensin II.

The contribution of estrogen receptors to the regulation of vascular function in aging is another key area to examine. Although ERs are known to affect the vasculature in several ways, many questions remain regarding the roles of the ER α and β subtypes, intracellular versus membrane bound receptors, and their genomic versus nongenomic

mechanisms of action. Our cell culture data indicated that estrogen was reducing eicosanoid production in an ER-dependent manner since the ER antagonist, tamoxifen, reversed the inhibitory effect of estrogen. However, this hormone-receptor interaction could be investigated in much more detail. When specific inhibitors of the ER subtypes become available, it would be interesting to differentiate the effects of the two receptors in an endothelial cell culture system. Specifying the effect of each receptor subtype on endothelial metabolism would provide insight into the regulation of vasoactive pathways.

Examining the role of membrane bound ERs is another project that could expand on our previous cell culture study. Blocking membrane bound ERs with specific antibodies would reveal any membrane bound receptor-dependent effects of estrogen on the formation of vasoactive products such as eicosanoids and NO. If inhibition of the membrane bound ERs alters product formation, the mechanism could be further elucidated by repeating the experiments in the presence of transcription and translation blockers. This would indicate whether the receptors were acting genomically or simply altering cellular properties such as ion channel activity and pH.

Estrogen could also be acutely applied to isolated vessels in the presence or absence of membrane bound receptor-antibody. Because estrogen has acute effects on vessel function, blocking the receptors with antibodies would demonstrate the degree to which acute doses of estrogen are mediated through membrane bound ERs. Studies such as this should be conducted in both the estrogen-replaced and chronic PGHS-2 inhibition models mentioned above. The estrogen-replacement model can demonstrate the effect of both aging and chronic estrogen exposure on vascular responsiveness to acute doses of estrogen. It is possible that long-term exposure to estrogen changes ER expression and

therefore affects vessel sensitivity to acute doses of the hormone. Likewise, the chronic PGHS-2 inhibition model would reveal any effects that the pathway has on acute responses to estrogen.

The distribution of ERs in various tissues should be analyzed in young and aged vessels as well. Determining ER α and β expression in different vascular beds and how it is affected by aging may advance our understanding of the influence of estrogen and aging on the vasculature.

4.5. CONCLUSION

The results of our projects have complimented previous work by further specifying mechanisms regarding estrogen and aging. At a functional level, Davidge *et al.*[10] previously demonstrated estrogen's ability to suppress PGHS-dependent vasoconstriction; however, the nature of the experiment was not conducive to analyzing the cellular mechanism(s) involved. Therefore, in contrast to functional data, our cell culture study provides insight into the effect of estrogen at the level of product formation. In our model, we have concluded that estrogen decreases PGHS-dependent vasoconstriction by suppressing the production of vasoconstrictors such as TXA_2 and possibly PGH_2. This may represent one of the mechanisms through which estrogen decreases the incidence of cardiovascular disease in aging women.

Characterizing the vascular changes that occur during aging is an important precursor in developing therapeutic approaches to reduce the heightened risk of cardiovascular disease in postmenopausal women. We found that aging greatly reduced NO-mediated relaxation. However, our most significant finding with respect to aging is

likely the increase in PGHS-2-dependent vasoconstriction. This novel data presents a potentially new therapeutic target to reduce the incidence of cardiovascular disease in the aging population.

4.6. References

1. Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, Sudano I, Salvetti A. Aging and endothelial function in normotensive subjects and patients with essential hypertension. *Circulation*.1995;91:1981-7.

2. Rodriguez-Martinez MA, Alonso MJ, Redondo J, Salaices M, Marin J. Role of lipid peroxidation and the glutathione-dependent antioxidant system in the impairment of endothelium-dependent relaxations with age. *Br J Pharmacol*.1998;123:113-21.

3. Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y, Takeshita A. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation*.1993;88:77-81.

4. Kung CF, Luscher TF. Different mechanisms of endothelial dysfunction with aging and hypertension in rat aorta. *Hypertension*.1995;25:194-200.

5. Shirasaki Y, Su C, Lee TJ, Kolm P, Cline WH, Jr., Nickols GA. Endothelial modulation of vascular relaxation to nitrovasodilators in aging and hypertension. *J Pharmacol Exp Ther*.1986;239:861-6.

6. Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y, Fujishima M. Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat. *Hypertension*.1989;14:542-8.

7. Davidge ST, Hubel CA, McLaughlin MK. Impairment of vascular function is associated with an age-related increase of lipid peroxidation in rats. *Am J Physiol*.1996;271:R1625-31.

8. Johnson RD, Sadovsky Y, Graham C, Anteby EY, Polakoski KL, Huang X, Nelson DM. The expression and activity of prostaglandin H synthase-2 is enhanced in trophoblast from women with preeclampsia. *J Clin Endocrinol Metab*.1997;82:3059-62.

9. Auch-Schwelk W, Katusic ZS, Vanhoutte PM. Thromboxane A2 receptor antagonists inhibit endothelium-dependent contractions. *Hypertension*. 1990;15:699-703.

10. Davidge ST, Zhang Y. Estrogen replacement suppresses a prostaglandin H synthase-dependent vasoconstrictor in rat mesenteric arteries. *Circ. Res*.1998;83:388-395.

11. Node K, Kitakaze M, Kosaka H, Minamino T, Sato H, Kuzuya T, Hori M. Roles of NO and Ca2+-activated K+ channels in coronary vasodilation induced by 17 beta-estradiol in ischemic heart failure. *FASEB J*.1997;11:793-9.

12. Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, Bender JR. 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization. *Circ Res*.1997;81:885-92.

13. Geis GS. Update on clinical developments with celecoxib, a new specific COX-2 inhibitor: what can we expect? *Scand J Rheumatol Suppl*.1999;109:31-7.

14. Ho L, Pieroni C, Winger D, Purohit DP, Aisen PS, Pasinetti GM. Regional distribution of cyclooxygenase-2 in the hippocampal formation in Alzheimer's disease. *J Neurosci Res*.1999;57:295-303.

15. Babenko NA, Ruiz-Larrea MB, Martinez R, Martin C, Lacort M. Inhibition by estrogens of the oxidant-mediated mobilization of arachidonic acid in hepatocytes. *J Physiol Biochem*.1998;54:77-84.

16. Graber R, Sumida C, Vallette G, Nunez EA. Rapid and long-term effects of 17 beta-estradiol on PIP2-phospholipase C-specific activity of MCF-7 cells. *Cell Signal*.1993;5:181-6.

17. Periwal SB, Farooq A, Bhargava VL, Bhatla N, Vij U, Murugesan K. Effect of hormones and antihormones on phospholipase A_2 activity in human endometrial stromal cells. *Prostaglandins*.1996;51:191-201.

18. Morishita T, Nozaki M, Sano M, Yokoyama M, Nakamura G, Nakano H. Changes in phospholipase A_2 activity of the rabbit ampullary epithelium by ovarian steroids. *Prostaglandins Leukot Essent Fatty Acids*.1993;48:315-8.

19. Fayard JM, Chanal S, Felouati B, Macovschi O, Lagarde M, Pageaux JF, Laugier C. Regulation of quail oviduct phospholipase A2 activity by estradiol. *Eur J Endocrinol*.1994;131:205-12.

20. Phaneuf S, Europe-Finner GN, MacKenzie IZ, Watson SP, Lopez Bernal A. Effects of oestradiol and tamoxifen on oxytocin-induced phospholipase C activation in human myometrial cells. *J Reprod Fertil*.1995;103:121-6.

21. Cabot MC, Zhang Z, Cao H, Lavie Y, Giuliano AE, Han TY, Jones RC. Tamoxifen activates cellular phospholipase C and D and elicits protein kinase C translocation. *Int J Cancer*.1997;70:567-74.

22. Kulmacz RJ. Cellular regulation of prostaglandin H synthase catalysis. *FEBS Lett*.1998;430:154-7.

23. Kim YD, Farhat MY, Myers AK, Kouretas P, DeGroot KW, Pacquing A, Ramwell PW, Suyderhoud JP, Lees DE. 17-Beta estradiol regulation of myocardial glutathione and its role in protection against myocardial stunning in dogs. *J Cardiovasc Pharmacol*.1998;32:457-65.

24. Massafra C, De Felice C, Gioia D, Buonocore G. Variations in erythrocyte antioxidant glutathione peroxidase activity during the menstrual cycle. *Clin Endocrinol (Oxf)*.1998;49:63-7.

25. Ghanam K, Javellaud J, Ea-Kim L, Oudart N. The protective effect of 17 beta-estradiol on vasomotor responses of aorta from cholesterol-fed rabbit is reduced by inhibitors of superoxide dismutase and catalase. *Biochem Biophys Res Commun*.1998;249:858-64.

26. Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci U S A*.1996;93:15069-74.

27. Subbiah MT. Mechanisms of cardioprotection by estrogens. *Proc Soc Exp Biol Med*.1998;217:23-9.

28. Parfenova H, Balabanova L, Leffler CW. Posttranslational regulation of cyclooxygenase by tyrosine phosphorylation in cerebral endothelial cells. *Am J Physiol*.1998;274:C72-81.

29. Parfenova H, Fedinec A, Leffler CW. Role of tyrosine phosphorylation in the regulation of cerebral vascular tone in newborn pig in vivo. *Am J Physiol*.1999;276:H185-93.

30. Rosenstock M, Danon A, Rimon G. Prostaglandin H synthase: protein synthesis-independent regulation in bovine aortic endothelial cells. *Am J Physiol*.1997;273:C1749-55.

31. Ermert L, Ermert M, Althoff A, Merkle M, Grimminger F, Seeger W. Vasoregulatory prostanoid generation proceeds via cyclooxygenase-2 in noninflamed rat lungs. *J Pharmacol Exp Ther*.1998;286:1309-14.

32. Zerrouk A, Auguet M, Chabrier PE. Augmented endothelium-dependent contraction to angiotensin II in the SHR aorta: role of an inducible cyclooxygenase metabolite. *J Cardiovasc Pharmacol*.1998;31:525-33.

33. Ge T, Hughes H, Junquero DC, Wu KK, Vanhoutte PM, Boulanger CM. Endothelium-dependent contractions are associated with both augmented expression of prostaglandin H synthase-1 and hypersensitivity to prostaglandin H_2 in the SHR aorta. *Circ Res*.1995;76:1003-10.

34. Davidge ST, Hubel CA, McLaughlin MK. Cyclooxygenase-dependent vasoconstrictor alters vascular function in the vitamin E-deprived rat. *Circ Res*.1993;73:79-88.

35. Diederich D, Yang ZH, Buhler FR, Luscher TF. Impaired endothelium-dependent relaxations in hypertensive resistance arteries involve cyclooxygenase pathway. *Am J Physiol*.1990;258:H445-51.

36. Taddei S, Vanhoutte PM. Endothelium-dependent contractions to endothelin in the rat aorta are mediated by thromboxane A2. *J Cardiovasc Pharmacol*.1993;22 Suppl 8:S328-31.

37. Kato T, Iwama Y, Okumura K, Hashimoto H, Ito T, Satake T. Prostaglandin H2 may be the endothelium-derived contracting factor released by acetylcholine in the aorta of the rat. *Hypertension*.1990;15:475-81.

38. Boyer CS, Bannenberg GL, Neve EP, Ryrfeldt A, Moldeus P. Evidence for the activation of the signal-responsive phospholipase A_2 by exogenous hydrogen peroxide. *Biochem Pharmacol*.1995;50:753-61.

39. Chakraborti S, Chakraborti T. Down-regulation of protein kinase C attenuates the oxidant hydrogen peroxide-mediated activation of phospholipase A_2 in pulmonary vascular smooth muscle cells. *Cell Signal*.1995;7:75-83.

40. Marshall PJ, Kulmacz RJ, Lands WE. Constraints on prostaglandin biosynthesis in tissues. *J Biol Chem*.1987;262:3510-7.

41. Shitashige M, Morita I, Murota S. Different substrate utilization between prostaglandin endoperoxide H synthase-1 and -2 in NIH3T3 fibroblasts. *Biochim Biophys Acta*.1998;1389:57-66.

42. Ye SM, Johnson RW. Increased interleukin-6 expression by microglia from brain of aged mice. *J Neuroimmunol*.1999;93:139-48.

43. Prechel MM, Halbur L, Devata S, Vaidya AM, Young MR. Increased interleukin-6 production by cerebral cortical tissue of adult versus young mice. *Mech Ageing Dev*.1996;92:185-94.

44. Belmin J, Bernard C, Corman B, Merval R, Esposito B, Tedgui A. Increased production of tumor necrosis factor and interleukin-6 by arterial wall of aged rats. *Am J Physiol*.1995;268:H2288-93.

45. Nakagawa T, Fujita N, Oh-Hara T, Kurokawa T, Nakamura K, Tsuruo T. Interleukin-1 alpha induced cyclooxygenase-2 expression in bone-derived endothelial cells. *J Cell Physiol*.1999;179:226-32.

46. Vlahos R, Stewart AG. Interleukin-1alpha and tumour necrosis factor-alpha modulate airway smooth muscle DNA synthesis by induction of cyclo-oxygenase-2: inhibition by dexamethasone and fluticasone propionate. *Br J Pharmacol*.1999;126:1315-24.

47. Bany BM, Kennedy TG. Role of interleukin 1 in the regulation of cyclooxygenase gene expression in rat endometrial stromal cells. *J Reprod Fertil*.1999;115:125-31.

48. Peri KG, Varma DR, Chemtob S. Stimulation of prostaglandin G/H synthase-2 expression by arachidonic acid monoxygenase product, 14,15-epoxyeicosatrienoic acid. *FEBS Lett*.1997;416:269-72.

49. Coroneos EJ, Kester M, Maclouf J, Thomas P, Dunn MJ. Calcium-regulated protein tyrosine phosphorylation is required for endothelin-1 to induce prostaglandin endoperoxide synthase-2 mRNA expression and protein synthesis in mesangial cells. *J Am Soc Nephrol*.1997;8:1080-90.

50. Kester M, Coroneos E, Thomas PJ, Dunn MJ. Endothelin stimulates prostaglandin endoperoxide synthase-2 mRNA expression and protein synthesis through a tyrosine kinase-signaling pathway in rat mesangial cells. *J Biol Chem*.1994;269:22574-80.

51. Schramek H, Coroneos E, Dunn MJ. Interactions of the vasoconstrictor peptides, angiotensin II and endothelin-1, with vasodilatory prostaglandins. *Semin Nephrol*.1995;15:195-204.

52. Coroneos E, Kester M, Thomas P, Dunn MJ. Endothelin regulates PGE_2 formation in rat mesangial cells through induction of prostaglandin endoperoxide synthase-2. *Adv Prostaglandin Thromboxane Leukot Res*.1995;23:117-9.

53. Komatsumoto S, Nara M. Changes in the level of endothelin-1 with aging. *Nippon Ronen Igakkai Zasshi*.1995;32:664-9.

54. Battistelli S, Gori s, Borgogni T, Manasse G. Variation in the plasma endothelin levels in relation to age. *Minerva Cardioangiol*.1996;44:111-4.

55. Sato I. Alteration in production of vasomediators associated with in vitro aging in cultured human umbilical vein endothelial cells. *Kokubyo Gakkai Zasshi*.1994;61:427-36.

56. Zakar T, Mijovic JE, Eyster KM, Bhardwaj D, Olson DM. Regulation of prostaglandin H2 synthase-2 expression in primary human amnion cells by tyrosine kinase dependent mechanisms. *Biochim Biophys Acta*.1998;1391:37-51.

57. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*.1996;271:C1424-37.

58. Komhoff M, Grone HJ, Klein T, Seyberth HW, Nusing RM. Localization of cyclooxygenase-1 and -2 in adult and fetal human kidney: implication for renal function. *Am J Physiol*.1997;272:F460-8.

59. Thore CR, Beasley TC, Busija DW. In vitro and in vivo localization of prostaglandin H synthase in fetal sheep neurons. *Neurosci Lett*.1998;242:29-32.

60. Park JM, Yang T, Arend LJ, Smart AM, Schnermann JB, Briggs JP. Cyclooxygenase-2 is expressed in bladder during fetal development and stimulated by outlet obstruction. *Am J Physiol*.1997;273:F538-44.

61. Asano K, Lilly CM, Drazen JM. Prostaglandin G/H synthase-2 is the constitutive and dominant isoform in cultured human lung epithelial cells. *Am J Physiol*.1996;271:L126-31.

62. Cullen L, Kelly L, Connor SO, Fitzgerald DJ. Selective cyclooxygenase-2 inhibition by nimesulide in man. *J Pharmacol Exp Ther*.1998;287:578-82.

63. Tomasoni S, Noris M, Zappella S, Gotti E, Casiraghi F, Bonazzola S, Benigni A, Remuzzi G. Upregulation of renal and systemic cyclooxygenase-2 in patients with active lupus nephritis. *J Am Soc Nephrol*.1998;9:1202-12.

64. Zhang F, Ram JL, Standley PR, Sowers JR. 17 beta-Estradiol attenuates voltage-dependent Ca^{2+} currents in A7r5 vascular smooth muscle cell line. *Am J Physiol*.1994;266:C975-80.

65. Hishikawa K, Nakaki T, Marumo T, Suzuki H, Kato R, Saruta T. Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells. *FEBS Lett*.1995;360:291-3.

66. Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, Pick S, Trzaskos JM. Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase. *Proc Natl Acad Sci U S A*.1994;91:11202-6.

67. Li Z, Froehlich J, Galis ZS, Lakatta EG. Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. *Hypertension*.1999;33:116-23.

68. Robert V, Besse S, Sabri A, Silvestre JS, Assayag P, Nguyen VT, Swynghedauw B, Delcayre C. Differential regulation of matrix metalloproteinases associated with aging and hypertension in the rat heart. *Lab Invest*.1997;76:729-38.

69. Nelson MT, Huang Y, Brayden JE, Hescheler J, Standen NB. Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. *Nature*.1990;344:770-3.

70. Cheng DY, Gruetter CA. Chronic estrogen alters contractile responsiveness to angiotensin II and norepinephrine in female rat aorta. *Eur J Pharmacol*.1992;215:171-6.

71. Ravi J, Mantzoros CS, Prabhu AS, Ram JL, Sowers JR. In vitro relaxation of phenylephrine- and angiotensin II-contracted aortic rings by beta-estradiol. *Am J Hypertens*.1994;7:1065-9.

72. Gallagher PE, Li P, Lenhart JR, Chappell MC, Brosnihan KB. Estrogen regulation of angiotensin-converting enzyme mRNA. *Hypertension*.1999;33:323-8.

73. Tanaka M, Nakaya S, Watanabe M, Kumai T, Tateishi T, Kobayashi S. Effects of ovariectomy and estrogen replacement on aorta angiotensin- converting enzyme activity in rats. *Jpn J Pharmacol*.1997;73:361-3.

74. Brosnihan KB, Senanayake PS, Li P, Ferrario CM. Bi-directional actions of estrogen on the renin-angiotensin system. *Braz J Med Biol Res*.1999;32:373-81.

75. Toda N, Ayaziki K, Okamura T. Modifications by endogenous prostaglandins of angiotensin II-induced contractions in dog and monkey cerebral and mesenteric arteries. *J Pharmacol Exp Ther*.1990;252:374-9.

APPENDIX

Protein Assay:

Protein concentrations in cell culture and vessel homogenates was determined using the micro-Bradford method. A standard curve was constructed using doses of bovine serum albumin (0.25 μg/10μl to 4 μg/10μl). Concentrations of the standard (10 μl) and samples (10 μl) were added to a 96 well plate. 200 μl of Bio-Rad protein assay dye reagent (diluted 1:10) was added to each well. The plate was then analyzed in a UV max kinetic microplate reader (Molecular Devices) at 595 nm for determination of protein values.

Greiss Assay:

The Greiss assay was used to measure nitrite formation, an indicator of NO synthesis. A standard curve was constructed with a sodium nitrite solution (0.25μM to 32μM). Concentrations of the standard (180 μl) and samples (180 μl) were added to a 96 well plate. Greiss reagent (20μl) consisting of equal volumes of naphthylene diamine dihydrochloride (1g/100ml H_2O) and sulfanilamide (10g/100ml, consisting of 59ml 85% H_3PO_4 and H_2O) was then added to each well. The plate was then analyzed in a UV max kinetic microplate reader (Molecular Devices) at 550 nm for determination of nitrite levels.

Endothelial Cell Culture:

A bovine coronary microvascular endothelial cell line was obtained from Gensia (San Diego, CA). Since the establishment of this cell line, the phenotype of these endothelial cells has been maintained for over 180 passages. The cellular characteristics include growth as a monolayer, a cobblestone morphology at confluence, positive immunostaining for von Willebrand factor-related antigen, the presence of receptors for acetylated low-density lipoproteins (unpublished observation), and secretion of prostaglandins, NO, and tissue-type plasminogen activator.

Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2-95% air with alpha-modified minimum essential medium (GIBCO) (0.6 mM L-arginine) containing 10% horse serum, 2 mM L-glutamine, gentamicin (5 μg/ml), kanamycin (20 μg/ml), and nystatin (10 U/ml).

Cell cultures were dispersed with 0.05% trypsin-0.53 mM EDTA, plated in 6-well plates, and grown to confluent monolayers. Preliminary experiments, in which cell number was measured by a hemacytometer, demonstrated that each well contained approximately 10^6 cells at confluence. The consistency of the cell number of each well was confirmed by measuring protein content using the Bradford method and resulted in <5% variation.

Cell Collection:

At the time of collection, the supernatant from the bovine coronary microvascular endothelial cells was placed in microcentrifuge tubes and stored at –80 C for later measurement. Phosphate buffered saline (1ml/well) was used to rinse the cells for approximately 10 seconds and then removed. Homogenizing buffer (25 mM Tris-Cl, pH 7.5 with 0.5% triton X-100) (100 µl) was then added to each well and the cells were manually scraped free from the bottom surface of the 6-well plate. Another 100 µl of homogenizing buffer was then added to the wells and the cell suspension was collected into microcentrifuge tubes. Each tube of cells was sonicated for approximately 4-6 seconds while placed on ice.

Vessel Preparation:

A section of the mesentery 5 to 10 cm distal to the pylorus was rapidly removed and placed in ice-cold N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffered physiological saline solution (HEPES-PSS) (sodium chloride 142, potassium chloride 4.7, magnesium sulfate 1.17, calcium chloride 1.56, potassium phosphate 1.18, HEPES 10, and glucose 5.5 mmol/L, pH 7.4). Two mesenteric arteries averaging 250 µm in diameter were dissected free from surrounding adipose tissue, cut into two 1.8 mm lengths and threaded onto 20 µm wires. These wires were fastened to four stainless steel blocks that were mounted in a specially designed isometric myograph system. One block was attached to a Kent Scientific Corporation strain gauge force transducer (Kent Scientific Corporation, Litchfield, CT), the other connected to a displacement device. The blocks rested in 5 ml glass-jacketed organ

baths with HEPES-PSS solution kept at 37° C. Each experiment had four baths running simultaneously.

Resting Length-Tension Curve:

After mounting, the arteries were stretched to about 0.2 mN/mm vessel length (1mN = 102 mg) and allowed to equilibrate for 30 minutes in HEPES-PSS buffer. The arteries were then given a conditioning stretch of about 0.6 mN. To generate the resting curve, each artery was stretched in six incremental steps and held for 20 seconds at each step. The force at each stretch was read at the end of 20 seconds and the displacement was measured. These values determined the resting length-tension relationship. The arterial circumference that was used to perform the dose-response curves was obtained by using the Law of LaPlace. With this equation, L_{100} is calculated from the exponential curve fit of tension versus circumference. L_{100} is defined as the circumference the vessel would have at a transmural pressure of 100 mmHg. We have found from our previous studies that dose-response curves obtained at .8 L_{100} is a point that provides maximum active force generation with minimum passive tension.

The vessels were incubated in fresh HEPES-PSS for 30 minutes following the resting-curve stretch. Dose response curves were then conducted in each of the vessel baths.

Vessel Isolation and Homogenization:

A section of mesentery from the same experimental animals was rapidly removed, placed in liquid nitrogen and stored at -80^{o}C. Mesenteric arteries were later dissected free from the section of mesentery and any residual blood products were removed. Homogenization of mesenteric arteries was conducted in the presence of liquid nitrogen. Vessels were homogenized in a homogenizing buffer containing protease inhibitors (final concentrations: 1 M KCl, 1 M Tris-Cl, pH 6.8, 100mM ZnCl, 500 mM EDTA, 100 mM DTT, 4 mg/ml Pepstatin, 0.4 μg/ml Leupeptin, 2μg/ml Chymostatin, 3.5 mg/ml TPCK, 3.5 mg/ml TLCK, 250mM PMSF).

SDS-PAGE and Western Immunoblotting:

For gel electrophoresis, samples were diluted by addition of an equal volume of 2X gel sample buffer (40 mM Tris/Cl pH 6.8, 2% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.02 % bromphenol blue). Samples were boiled for 3 minutes. Equal protein (5μg) was loaded into individual wells formed within the stacking gel (30% acrylamide/ 0.8% bisacrylamide 0.65ml, 4X Tris-Cl/SDS, pH 6.8 1.25ml, H_2O 3.05 ml.) overlayed on 8 or 10% acrylamide gradient gels respectively (30% acrylamide/ 0.8% bisacrylamide 4 or 5 ml, 4X Tris-Cl/SDS, pH 8.8 3.75 ml, H_2O 7.25 or 6.25 ml, 10% ammonium persulfate 0.05 ml, temed 0.001 ml) in Tris-HCl, pH 8.8, and separated by electrophoresis at approximately 120 volts for ~1 h in a mini-gel apparatus (E-C Apparatus Corporation).

Following separation, samples were transferred to a membrane (Nylon NT, Magnagraph, Inc.) in a wet tank (vertical gel system, E-C Apparatus mini) at 15 volts for approximately 2 hours. Prestained standards (Bio-Rad Laboratories, Canada) were included in separate lanes in each gel for identification of the approximate molecular weight of unknowns. Following transfer, the membranes were incubated overnight in Tris-buffered saline (TBS: 50 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 5% non-fat dry milk. The following day the membranes were washed in TTBS (TBS, 0.1% Tween-20), 3 rinses, once for 15 minutes, and twice for 5minutes. The membrane was then incubated with mouse monoclonal antibodies, diluted 1:1000 in TBS, for 2 hours at room temperature. The above wash was repeated before addition of horseradish peroxidase labeled anti-mouse polyclonal antibody, diluted 1:2000, for 1 hour. The above wash was then repeated, followed by 4 rinses with 1X TBS. Immunoreactive bands were visualized by addition of ECL kit reagents (Amersham LIFE Science).